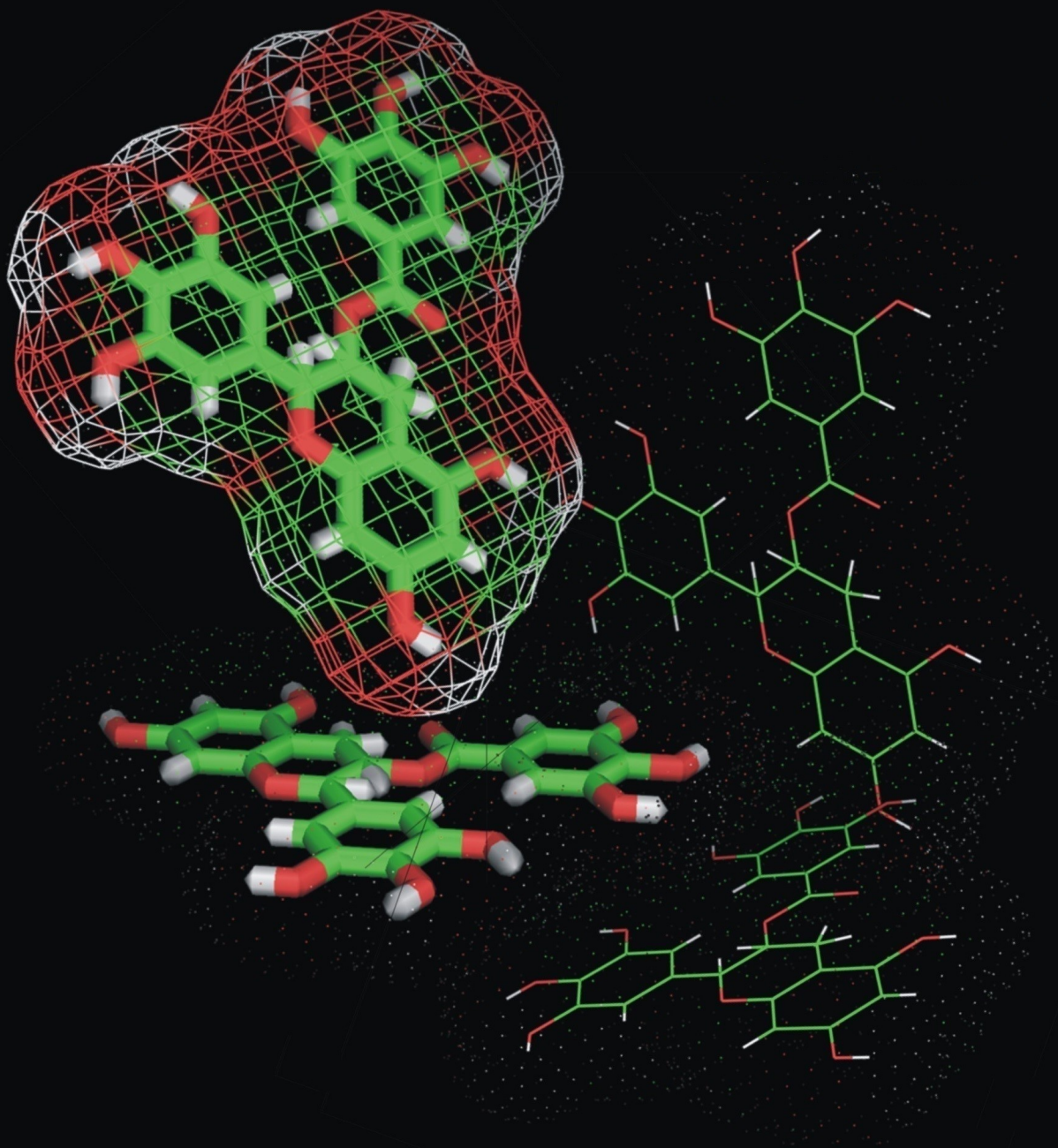
ISSN 2087-2852

The Journal of Experimental Life Science

Discovering Living System Concept through Nano, Molecular and Cellular Biology



J. Exp. Life. Sci.	Vol. 10	No. 1	pages. 1-71	February 2020

Published by

Graduate Program, University of Brawijaya
in Cooperation With
Masyarakat Nano Indonesia (MNI)

ISSN. 2087-2852
E-ISSN. 2338-1655

The Journal of Experimental Life Science

Discovering Living System Concept through Nano, Molecular and Cellular Biology

Editorial Board

Address
The Journal of Experimental Life Science
Building B, 1st Floor, Postgraduate School, University of Brawijaya
Jl. Mayor Jenderal Haryono 169, Malang, 65145
Telp: (0341) 571260 ; Fax: (0341) 580801
Email: jels@ub.ac.id
Web: http://www.jels.ub.ac.id

ISSN. 2087-2852
E-ISSN. 2338-1655

Table of Content

Evaluation of Proteolytic and Chitinolytic Activities of Indigenous Bacillus Species from Crab Shell Waste
(Moh Dliyauddin, Tri Ardyati, Suharjono Suharjono) 1-5
DOI: https://doi.org/10.21776/ub.jels.2019.010.01.01

Effect of Colchicine on In Vitro Growth and Ploidicity of Crown Vetiver Plant (*Vetiveria zizanioides* L. Nash)
(Eki Juanda, Sri Andayani, Maftuch Maftuch) 6-11
DOI: https://doi.org/10.21776/ub.jels.2019.010.01.02

Assessment of Water Quality Using Benthic Macroinvertebrate Along Sumber Maron River, District of Gondanglegi Kulon, East Java-Malang, Indonesia
(Faiza Tawati, Yenny Risjani, M Sasmito Djati, Bagyo Yanuwiadi) 12-19
DOI: https://doi.org/10.21776/ub.jels.2019.010.01.03

Effect of Benzyl Adenine Concentration on Callus Induction of Geranium Plants (*Pelargonium graveolens* L'Her) from Petiole and Leaf Explants
(Moch Faizul Huda, Serafinah Indriyani, Wahyu Widoretno) 20-23
DOI: https://doi.org/10.21776/ub.jels.2019.010.01.04

Detection of Reproductive Status in Ongole Crossbred (PO) Cow Based On Vaginal Epithel Morphology and Profile Hormone
(Yeni Widyaningrum, Aulanni'am Aulanni'am, Agung Pramana Warih Marhendra) 24-28
DOI: https://doi.org/10.21776/ub.jels.2019.010.01.05

The Artificial Bee Colony (ABC) Algorithm for Estimating Parameter of Epidemic Influenza Model
(Ririn Nirmalasari, Agus Suryanto, Syaiful Anam) 29-36
DOI: https://doi.org/10.21776/ub.jels.2019.010.01.06

Combination *Moringa oleifera* Extract and Ifalmin as Potential Formulation of Preventing Inflammation in Diabetic Mice Model
(Ruri Vivian Nilamsari, Wahyu Isnia A, Noviana Dwi Lestari, Hideo Tsuboi, Muhaimin Rifa'i) 37-42
DOI: https://doi.org/10.21776/ub.jels.2019.010.01.07

The Role of *Moringa oleifera*- Ifalmin® Formulation in Regulation of B220+IgM+ and B220+IgG+ in Diabetic Mice
(Wahyu Isnia A, Ruri Vivian Nilamsari, Noviana Dwi Lestari, Nashi Widodo, Muhaimin Rifa'i) 43-48
DOI: https://doi.org/10.21776/ub.jels.2019.010.01.08

Physiological and Genetic Variations of Amorphophallus variabilis in Bojonegoro based on Glucomannan Content, Calcium Oxalate and RAPD Markers
(Achmad Yogi Pambudi, Nunung Harijati, Estri Laras Arumingtyas) 49-54
DOI: https://doi.org/10.21776/ub.jels.2019.010.01.09

Repairing Cell Structure of Jejunum Tissue in RA-CFA Rat Model Improved by Caprine CSN1S2 Protein
(Laili Nur Zaidah, Aris Soewondo, Fatchiyah Fatchiyah) 55-60
DOI: https://doi.org/10.21776/ub.jels.2019.010.01.10

The Effectiveness of Combination of Momordica charantia Extract and Averrhoa bilimbi on Nf-Kb Activation in Mice (*Mus musculus*) Balb/C Hyperglycemia Models
(Intan Sartika Risky Sampurna, Muhaimin Rifa'I, Sri Rahayu) 61-64
DOI: https://doi.org/10.21776/ub.jels.2019.010.01.11

Physicochemical Characteristics and Consumer Acceptance of Bagged Indonesian Green Tea (*Camellia sinensis*) Formulated with Cinnamon Bark (*Cinnamomum burmannii*) and Lemon (*Citrus limon*) Peel
(Adinda Kirana, Wenny Bekti Sunarharum) 65-71
DOI: https://doi.org/10.21776/ub.jels.2019.010.01.12

Evaluation of Proteolytic and Chitinolytic Activities of Indigenous *Bacillus* Species from Crab Shell Waste

Moh Dliyauddin[1*], Tri Ardyati[2], Suharjono[2]

[1]Master Program of Biology, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia
[2]Department of Biology, Faculty of Mathematicsand Natural Sciences, University of Brawijaya, Malang, Indonesia

Abstract

The increase of crab meat export activities in Indonesia leads to the accumulation of crab shell waste in a massive amount that can naturally degrade and causing bad odor. Naturally, microorganisms will degrade this waste through fermentation and enzymatic reaction, including protease and chitinase due to its high content of chitin and protein. *Bacillus* is the most potential bacteria to degrade crab shell waste, and indigenous *Bacillus* from this waste can increase the degradation rates. The aims of this study were to evaluate the proteolytic and chitinolytic activities of indigenous *Bacillus* species from crab shell waste. *Bacillus cereus* BP14 and *Bacillus licheniformis* CK20 as the chitinolytic bacteria, and *Bacillus subtilis* AP9 and *Bacillus licheniformis* AP5 as the proteolytic bacteria were isolated from crab shell waste and identified based on the 16S rDNA sequences. The bacterial isolates were grown in skim milk broth for proteolytic characterization and colloidal chitin broth for chitinolytic characterization. The growth rates of each bacteria were determined through the growth curves. The enzymatic activities were determined based on the international standard for protease and chitinase enzyme activities together with growth curve sampling to determine the best incubation time for obtaining the highest enzymatic activities. From the shortest to the longest generation times of the *Bacillus* species obtained were *B. subtilis* AP9, *B. licheniformis* CK20, *B. cereus* BP14, and *B. licheniformis* AP5, respectively. The best incubation time for producing the highest enzymatic activity varied among species. However, the end of the logarithmic phase was similar. All of the *Bacillus* species obtained from this study exhibited chitinolytic and proteolytic activity. Therefore, it can be used as promising candidates of biodegradation agents inenhancing the degradation rates of crab shell wastes.

Keywords: *Bacillus cereus, Bacillus licheniformis*, *Bacillus subtilis*, chitinolytic, crab shell waste, proteolytic.

INTRODUCTION

The high demand for crab meat export in Indonesia causes the accumulation of crab shell wastes in the environment. One of the third most valuable marine commodities for export is blue swimming crab (BSC) or locally named as Rajungan crab (*Portunuspelagicus*) [1]. The effort to improve the BSC stock in Indonesia's fisheries are also developed in recent years so that the demand for its export can be fulfilled [2]. However, it is not followed by the improvement of waste management, including shell waste.

In Indonesia, the crab shell wastes are usually left on the open-terrain without processing. It will next naturally fermented by microorganisms and causing the bad odor, which pollutes the air in the environment. Thus, the research on the utilization of this waste in recent years is developed. Crab shell waste can be used to produce natural flavor powder [3], while this shell waste along with shrimp waste, can be used to produce N-Acetylglucosamine by chitinolytic soil *Streptomyces* sp. SJKP9 [4]. The utilization of this waste can improve the economic value of waste. However, due to the high cost of this processing management, it cannot be applied in Indonesia, especially in the marine fishery regions which the economy is middle to low and the technology is still poor.

Because of this reason, the possible effort to manage the crab shell wastes left in the fishery regions is by enhancing its degradation rates, so the time needed for the fermentation or degradation can be reduced. It can be done by inoculating microorganisms, especially the indigenous microorganism, which can produce protease and chitinase enzymes with high activity for crab shell wastes degradation. The crab shell waste can be used as the resource of chitin that can be utilized by microorganisms to grow and produce chitosan, including water-soluble chitosan [5], that can be further used in the biomedical and pharmaceutical application [6]. This study aimed to determine the proteolytic and chitinolytic activity of indigenous *Bacillus* species from crab shell wastes for obtaining promising candidate inoculums to enhance the degradation rates.

*Correspondence address:
Moh Dliyauddin
Email : m.dliyauddin.md@gmail.com
Address : Dept. Biology, Brawijaya University, Malang 65145

MATERIAL AND METHOD

Bacterial Isolates

The bacterial isolates used in this study were *Bacillus subtilis* AP9 and *B. licheniformis* AP5 as proteolytic bacteria, while *B. cereus* BP14 and *B. licheniformis* CK20 as chitinolytic bacteria. All strains were isolated from blue swimming crab shell wastes using skim milk agar (casein 0.5%, yeast extract 0.25%, dextrose 0.1%, skim milk 2.8%, agar 1.5%) [7] and colloidal chitin agar medium (bacto agar 1.5%, yeast extract 0.5%, NH_4Cl 0.6%, Na_2HPO_4 0.6%, KH_2PO_4 0.3%, NaCl 0.1%, colloidal chitin 0.5%.

Growth Curves Preparation and Extracellular Enzymes Production

The proteolytic bacterial strains were grown in skim milk broth (5 $g.L^{-1}$ casein, 2.5 $g.L^{-1}$ yeast extract, 1 $g.L^{-1}$ glucose, 1 $g.L^{-1}$ skim milk) [8] for 48 h of incubation in a rotary shaker incubator at room temperature. Meanwhile, the chitinolytic bacterial strains were grown in colloidal chitin broth for 48 h of incubation in rotary shaker incubator at room temperature. The sampling was conducted every 4 h by taking 3 mL of culture and storing it in the sterile tubes. It was then 1 mL used for enumerating the cell number using a haemocytometer to determine the generation times and growth phases. While the rest volume (2 mL) was centrifuged on 10.000 rpm at 4°C for 10 min and the pellets were discarded. The supernatant was assumed as the crude extracellular enzyme for both protease and chitinase. The crude extracellular enzymes were stored on the 4°C for further assay.

Proteolytic and Chitinolytic Assay

The crude enzymes were evaluated for the chitinolytic and proteolytic activities using standard methods. For proteolytic characterization, 1% casein solution was used as the substrate for protease activity that follows the standard procedure of Sigma [9] with few modifications. The crude was reacted with 1% casein solution and incubated at 37°C for 20 min, and the reaction was stopped by the addition of 110 mM Trichloroacetic acid (TCA), and the result was measured using UV-Vis spectrophotometer (Thermo Spectronic, New York, AS) at 540 nm. The L-tyrosine standard curve was also prepared to determine the enzymatic activity (U mL^{-1}). One unit of protease enzyme activity is defined as the amount of enzyme used to hydrolyze 1 µg substrate per min [9].

For chitinolytic characterization, the crude enzyme was reacted with 1% colloidal chitin as a substrate and incubated at 30°C for 2 h [10]. The mixture was added with 3,5-dinitrosalicylic acid (DNS) reagent then heated at 100°C for 5 min before measured using UV-Vis spectrophotometer at 540 nm. The N-acetylglucosamine standard curve was also prepared to determine the chitinolytic activity. One unit chitinase enzyme activity is assumed as the total of the enzyme for hydrolyzing 1 µg substrate per h [10].

Data analysis

Data of growth were analyzed based on One Way Analysis of Variance (ANOVA). Data of proteolytic and chitinolytic activity were completed randomized design analyzed with Multivariate Analysis of Variance (MANOVA) using SPSS 16.0 application for windows with ρ value ≤ 0.05.

RESULTS AND DISCUSSION

Growth curves and generation times

A total of 20 proteolytic bacteria isolates and 22 chitinolytic bacteria isolates were successfully isolated from the samples and have the potential to produce protease and chitinase enzymes. The results showed that the growth phases of each potential bacteria strain were varied. However, all of the strains achieved their logarithmic phase (log) on 32 h of incubation time (Fig. 1) and then followed by a constant growth (also called stationary phase) until 48 h. Based on the growth phases obtained from this study, the generation time of each *Bacillus* strain can be determined. The generation time from the fastest to slowest were 4.04 h, 6.77 h, 9.45 h, and 11.88 h of *B. subtilis* AP9, *B. licheniformis* CK20, *B. cereus* BP14, and *B. licheniformis* AP5, respectively (Table 1).

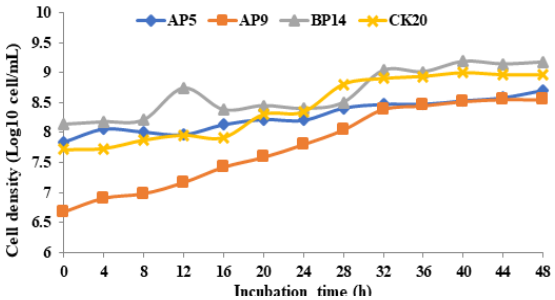


Figure 1. Growth curves of *Bacillus* species

Table 1. The generation times of *Bacillus* species

No.	Strains	Generation time (h)
1.	*Bacillus subtilis* AP9	4.04
2.	*Bacillus licheniformis*CK20	6.77
3.	*Bacillus cereus* BP14	9.45
4.	*Bacillus licheniformis*AP5	11.88

ISSN. 2087-2852
E-ISSN. 2338-1655

In a normal condition with high nutritious medium content, *Bacillus* species generation time is 20-30 min [11], while in the lack of nutrients, the generation time is varied from 1-14 h. For example, *B. amyloliquefaciens* BSM-1 was 4.66 h and *B. amyloliquefaciens* BSM-2 was 5.25 h in medium supplemented with low-density polyethylene (LDPE) for biodegradation purpose [12]. The variation of generation times can be caused by the nutrition contents in the growth medium. Interestingly, the generation time of *B. licheniformis* CK20 and *B. cereus* BP14, which were grown in colloidal chitin broth medium that contained complex energy source (chitin), was faster than of *B. licheniformis* AP5 which was grown in skim milk broth medium. It indicated that *B. licheniformis* CK20 and *B. cereus* BP14 are potential candidates for crab shell wastes degradation due to their ability to degrade and utilize chitin in a short period. Microorganism requires nutrients to grow and maintain their life. The most microorganism will metabolize simple and complex sugars, including cellulose [13].

Protease and Chitinase Activity of *Bacillus* Species

Proteolytic and chitinolytic activities produced by each potential isolate have different variations at different incubation times (Fig. 2 and 3). Each potential isolate was able to produce proteolytic and chitinolytic enzymes simultaneously. Based on the results of the completed randomized design analysis, it was shown that proteolytic and chitinolytic activity significantly associated with incubation time and potential isolates ($p \leq 0.05$).

The AP9 isolate had the highest activity (32.03 ± 0.47 Unit.mL^{-1}) compared to other isolates with an incubation time of 44 hours. Meanwhile, the highest protease activity of AP5 and BP14 isolates produced at 44 hours incubation time was 26.24 ± 0.26 Unit.mL^{-1} and 17.94 ± 1.18 Unit.mL^{-1} of enzymes per minute, respectively. The isolate of CK20 produced the highest protease activity by 24.62 ± 1.05 Unit.mL^{-1} of enzymes per minute at 48 hours of incubation.

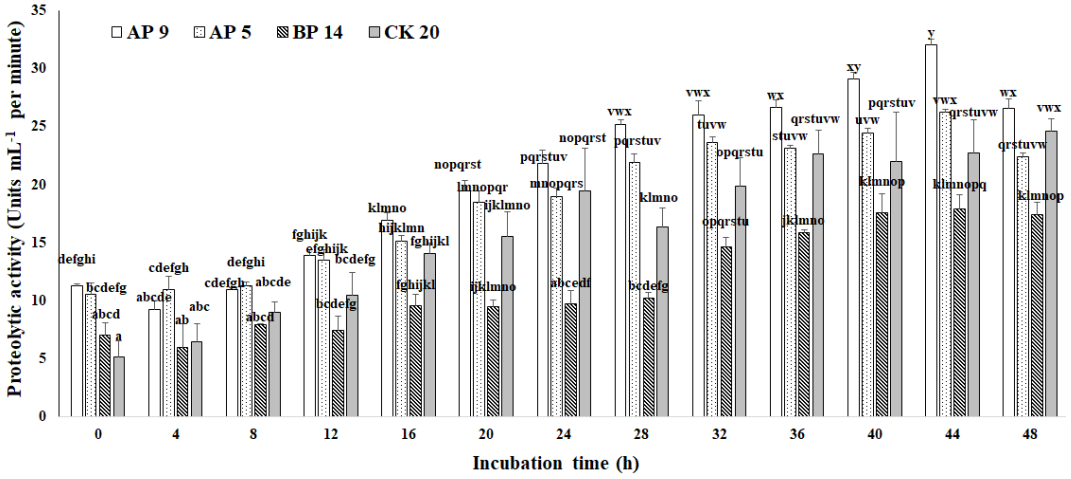


Figure 2. Proteolytic activity of potential isolates

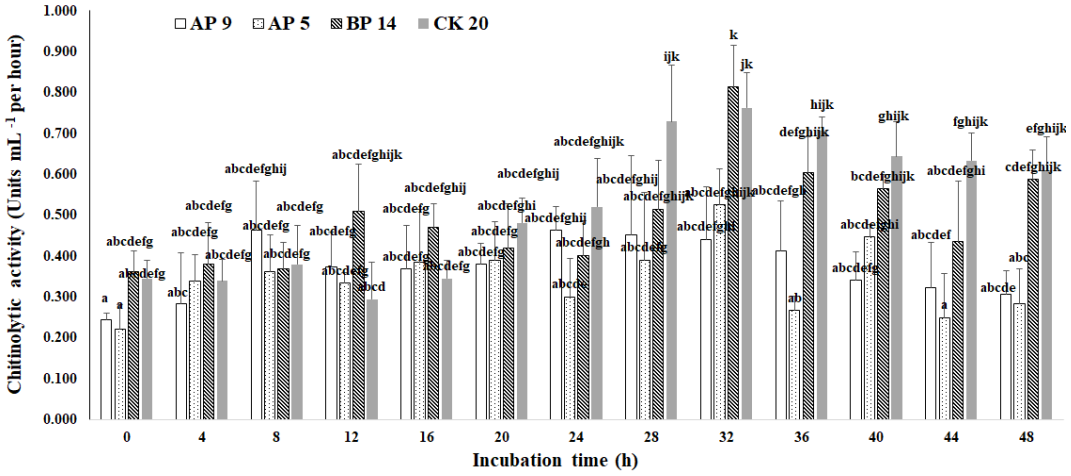


Figure 3. Chitinolytic activity of potential isolates

ISSN. 2087-2852
E-ISSN. 2338-1655

The best chitinase activity was shown by BP14 isolate with the chitinolytic activity of 0.813 ± 0.10 Unit.mL^{-1} of enzymes per hour at 32 hours of incubation compared to other potential isolates. The highest chitinase AP5 isolate activity produced at 32 hours incubation time was 0.526 ± 0.08 Unit.mL^{-1} enzyme per hour, AP9 isolate the highest chitinase activity at 24 hours of incubation was 0.463 ± 0.05 Unit.mL^{-1} enzyme per hour, and CK20 isolate has the highest chitinase activity at 32hours of incubation, amounting to 0.763 ± 0.08 Unit.mL^{-1} of enzymes per hour.

The highest enzymatic activities of both protease and chitinase were produced after 30 hours of incubation time, which is a phase of bacterial growth ending the logarithmic phase. In the stationary growth phase, the activity of the enzymes produced is the same and not significantly different due to small and equal cell growth ($p > 0.05$). It indicated the highest enzymatic activity of *Bacillus* strains used in this study was reached at the end of the log phase or at the initial of the stationary phase. Protease enzyme of bacteria is produced during the beginning of the stationary phase to the middle of the stationary phase [14]. Meanwhile, the chitinase enzyme is produced during the middle of the log phase to the stationary phase [15].

Protease enzymes are secreted in the early bacterial growth phase entering stationary until the middle of the stationary phase [14], while the chitinase enzyme is produced in the mid-logarithmic phase until the stationary phase [15]. Protease and chitinase enzymes are the results of primary metabolites by bacteria, so the protease and chitinase enzymes produced are used by bacteria for their growth, which are supported by a large number of cells in the final logarithmic phase and the stationary phase of bacterial growth.

In this study, the optimization of pH and temperature to obtain the best enzymatic activity was not conducted yet. The proteolytic activity was categorized lower than the previous study. *Bacillus subtilis* can produce protease enzyme activity up to 243.28 Unit.mL^{-1} enzymes per h at 36 h incubation time on 45°C which has pH 10 [16]. The chitinolytic activity was categorized higher than the previous study. *Acinetobacter johnsonii* PBK2 and *B. amyloliquefaciens* SA1.2 have an activity of 0.213 mL^{-1} units at pH 7 at a temperature of 30°C [17]. Thus, it is necessary to determine the pH and temperature optimum conditions for obtaining the best enzymatic activity before applying it directly in the environment. By combining protease and chitinase enzymes, the degradation rates of chitin fibers and protein in crab shell wastes can be enhanced.

Bacillus species used in this study are potential candidates for biodegradation agents. In addition, these strains can be used to produce extracellular protease enzymes and chitinase for industrial-scale by degrading waste, which can improve the economic value. However, further study to optimization activity and characterization enzyme is still needed.

CONCLUSION

Indigenous *Bacillus* species from crab shell wastes were potential biodegradation agents due to their ability to produce high enzymatic activities, especially for the proteolytic and chitinolytic. This ability is still can be enhanced by the optimization for pH and temperature on their growth medium conditions.

REFERENCES

[1] Ghofar, A., S. Redjeki, H. Madduppa, M. Abbey, N. Tasunar. 2018. Inclusive blue swimming crab fishery management initiative in Betahwalang Demak, Indonesia. *IOP Conference Series: Earth and Environmental Science*. 1(116). 012068.

[2] Hamid, A., D.T. F. Lumban Batu, E. Riani, Y. Wardiatno. 2016. Reproductive biology of blue swimming crab (*Portunuspelagicus* Linnaeus, 1758) in Lasongko Bay, Southeast Sulawesi-Indonesia. *AACL Bioflux.* 9(5). 1053-1066.

[3] Mulyadi, A.F., J.M. Maligan, W. Wignyanto, R. Hermansyah. 2013. Organoleptic characteristics of natural flavour powder from waste of swimming blue crabs (*Portunus pelagicus*) processing: study on dextrin concentration and drying temperature. *Jurnal Teknologi Pertanian.* 14(3). 183-192.

[4] Jagadeeswari, S., M.D. Balakumaran, P. Vidya, P.T. Kalaichelvan, K.P. Selvam. 2012. Utilization of shrimp and crab wastes for the production of N- Acetylglucosamine by chitinolytic soil *Streptomyces* sp. SJKP9. *Dyn. Biochem. Process Biotechnol. Mol. Biol*. 6(2). 46-48.

[5] Lestari, P.I., I. Ulfin, H. Harmami, S. Suprapto, Y.L. Ni'mah. 2018. Water-soluble chitosan from waste swimming crab shell (*Portunus pelagicus*). *AIP Conference Proceedings*. 2049(1). 020085.

ISSN. 2087-2852
E-ISSN. 2338-1655

[6] Sarbon, N.M., S. Sandanamsamy, S.F.S. Kamaruzaman, F. Ahmad. 2015. Chitosan extracted from mud crab (*Scylla olivicea*) shells: physicochemical and antioxidant properties. *J. Food Sci. Technol*. 52(7). 4266-4275.

[7] Tennalli, G., B. Udapudi, P. Naik. 2012. Isolation of proteolytic bacteria and characterization of their proteolytic activity. *Int. J. Adv. Eng. Sci. Technol*. 2(3). 185-192.

[8] Fulzele, R., E. Desa, A. Yadav, Y. Shouche, R. Bhadekar. 2011. Characterization of novel extracellular protease produced by marine bacterial isolate from the Indian Ocean. *Braz. J. Microbiol*. 42(4). 1364-1373.

[9] Cupp-Enyard, C. 2008. Sigma's non-specific protease activity assay - casein as a substrate. *J. Vis. Exp*. 17(19). 899. DOI: 10.3791/899.

[10] Zarei, M., S. Aminzadeh, H. Zolgharnein, A. Safahieh, M. Daliri, K.A. Noghabi. 2011. Characterization of a chitinase with antifungal activity from a native *Serratia marcescens* B4A. *Braz. J. Microbiol*. 42(3). 1017-1029.

[11] Choi, H.J., S.J. Kang, K.W. Hong. 2017. Comparison of NheA toxin production and doubling time between Bacillus cereus and *Bacillus thuringiensis*. *Appl. Biol. Chem*. 60(5). 545-551.

[12] Das, M.P., S. Kumar, 2013. A comparative study on microbial growth and substrate utilization kinetics. *Int. J. Pharm. Technol*. 12(5). 5567-5573.

[13] Sandle, T. 2016. Microbiological challenges to the pharmaceuticals and healthcare. Pharmaceutical Microbiology. Oxford: Woodhead Publishing. 281–294.

[14] Ibrahim, A.S.S., A.A. Al-Salamah, Y.B. Elbadawi, M.A. El-Tayeb, S.S.S. Ibrahim. 2015. Production of extracellular alkaline protease by new halotolerant alkaliphilic *Bacillus* sp. NPST-AK15 isolated from hyper saline soda lakes. *Electron. Journal of Biotechnology*. 18(3). 236-243.

[15] Kumar, A., N. Gupta, Laksh, S. Angural, M. Rana. 2017. Process optimization of extracellular chitinase production from *Bacillus* sp. isolated from fish waste dumping site. *Eur. J. Pharm. Med. Res*. 8(4). 474-480.

[16] Pant, G., A. Prakash, J.V.P. Pavani, S. Bera, G.V.N.S. Deviram, A. Kumar. 2015. Production, optimization and partial purification of protease from *Bacillus subtilis*. *J. Taibah Univ. Sci*. 9(1). 50-55.

[17] Setia, I.N., Suharjono. 2015. Chitinolytic assay and identification of bacteria isolated from shrimp waste based on 16S rDNA sequences. *Adv. Microbiol*. 5. 541-548.

ISSN. 2087-2852
E-ISSN. 2338-1655

Effect of Colchicine on In Vitro Growth and Ploidicity of Crown Vetiver Plant (*Vetiveria zizanioides* L. Nash)

Anisa Firmandanur Sinta, Wahyu Widoretno*

Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang

Abstract

The goal of this research was to evaluate the effect of colchicine on the formation and growth of shoot from explant crowns by in vitro, as well as to obtain polyploidy vetiver plant (*Vetiveriazizanioides* L. Nash). Induction of polyploidy vetiver plants carried out by culturing explant crowns on MS media supplemented by 2 mg.L^{-1} BAP and colchicine (0, 30, 60, 90, 120 mg.L^{-1}) for three weeks. Explant and formed shoot regenerated on MS media containing 1 mg.L^{-1} NAA. The generated plantlets acclimatized on the growing media (cocopeat: husk charcoal: compost = 1: 1: 2). Plant ploidy level of the plants regenerated from treated explant estimated by chromosome counting in root tips. The effect of colchicine on media was able to induce polyploidy in vetiver plants. Five mixoploids were obtained from explant treated colchicine. The vetiver mixoploid plants obtained were 20% and 62.5% from colchicine 60 mg.L^{-1} and 90 mg.L^{-1}, respectively. The vetiver mixoploid plants consist of diploid (2n=2x=20) and triploid cells (2n=3x=30) or diploid (2n=2x=20) and tetraploid (2n=4x=40). The addition of colchicine in concentrations above 30 mg.L^{-1} caused inhibition of shoot formation and growth, even a concentration of 120 mg.L^{-1} caused explant death. However, 30 mg.L^{-1} colchicine could increase the number of formed shoots, while only 60 mg.L^{-1} and 90 mg.L^{-1} could be induced the mixoploidy in the vetiver plant. The results showed that colchicine treatment could increase ploidicity in vetiver plants in vitro, but caused inhibition of shoot formation and growth.

Keywords : Chromosome, colchicine, in vitro, polyploid, *Vetiveria zizanioides* L. Nash.

INTRODUCTION

Vetiver (*Vetiveria zizanioides* L. Nash) is a plant belonging to the Graminae group, which often used to produce vetiver oil essential oil. Vetiver oil is used as an ingredient in perfume and soap industry [1]. The world demands of vetiver oil reach 250-300 tons per year. However, Indonesia can only produce 75-200 tons per year from 2014-2017 [2,3].

Polyploid plants have several advantages, including wider leaves, larger stems and stomata diameters, and larger oil glands, which yield more oil production [4] as well as more bioactive compounds [5]. Polyploid plants were also used in the utilization of plant germplasm for high secondary metabolites production [6]. Polyploid *Echinacea purpurea* L. has higher caffeic acid derivates and alkamides than its diploid [7].

In vitro polyploid induction can be done by adding colchicine to the media [8,9]. Colchicine binds to tubulin protein to inhibit its polymerization and no spindle thread to form, which plays a critical role in cell division. Chromatids fail to reach the poles and continue into the interphase phase with double the number of chromosomes [10]. Polyploid induction with colchicine has been successfully carried out in *Pogostemon cablin* [4], vetiver grass [11], and ruzigrass [12]. This low production is caused by the limited availability of superior seeds, so there is a need to improve seedlings' quality and quantity. Therefore tissue culture techniques are widely used to produce superior plants, one of which is the induction of polyploid plants [6].

In vitro induction of polyploid vetiver with colchicine was carried out to determine the effect of colchicine on the growth and formation of shoots and to obtain polyploidy vetiver. This study is important to get superior seedling of vetiver plants with high essential oil content and in large quantities.

RESEARCH METHOD

Plant Material

Vetiver plants were obtained from Sengklek, Pamalayan Village, Bayongbong District, Garut, West Java. The shoots were harvested and surface sterilized with 96% alcohol for 1 minute and 50% bleach (5.25% NaClO) for 20 minutes and rinsed with sterile distilled water for 5 minutes twice. Explant crowns were isolated and cultured on MS media supplemented with 2 mg.L^{-1} BAP. Formed shoots were multiplied on MS media supplemented with 3 mg.L^{-1} BAP [13]. In vitro shoots were used as the initial explants.

* Correspondence address:
WahyuWidoretno
Email : wahyu_widoretno@yahoo.com
Address : Dept. Biology, University of Brawijaya, VeteranMalang, 65145.

ISSN. 2087-2852
E-ISSN. 2338-1655

Induction of Polyploid with Colchicine *In Vitro*

Explant crown from in vitro shoots was isolated and cultured on MS media supplemented with 2mg.L^{-1} BAP and different concentrations of colchicines (0, 30, 60, 90, and 120 mg.L^{-1}). Each treatment performed with eight replicates. Cultures were incubated at 24 ± 1°C for three weeks in light (600 lux). Percentage of survival explants, the percentage of explants that formed shoots, and the number of shoots formed were observed.

Treated explant and formed shoots were transferred to MS media supplemented with 3 mg.L^{-1} BAP without colchicine. Cultures incubated at a temperature of 24-25°C, in the light (600 lux) for three weeks.

Shoots that developed after three weeks of culture transferred on MS media with 1 mg.L^{-1} NAA for rooting. The cultures incubated at a temperature of 24-25°C, in the light (600 lux).

Plantlets were transferred into planting media (cocopeat: husk charcoal: compost = 1:1:2). Four weeks-old vetiver plants analyzed for its ploidy level. The ploidy level of plants was identified by chromosomes counting of the roots' tip.

Preparation of Chromosome and Estimation of Ploidy Level

Root-tips of the regenerated plant were pretreated in a 0.002 M hydroxyquinoline solution for 3 hours and fixed in ethanol-glacial acetic acid solution (3:1) at room temperature for an hour. The fixed root-tips were hydrolyzed in 1 N HCl at 65°C for 3 minutes. The root-tips then soaked for three days in a 2% acetoorcein dye solution. The dyed root-tips was placed on the glass slide and covered with a glass cover and then squashed.

Chromosomes were observed with an Olympus CX31 microscope with 1000x magnification. Ploidy level of the vetiver plants was estimated by chromosomal counting in root-tips. If 2n=20, then it is considered as diploid plantlets, whereas if the chromosomes are 3n=30 or doubles to 4n=40 or even more, then it is considered as polyploid.

Data Analysis

The research design used was a randomized block design with replications as a group. Data were analyzed by one way ANOVA (Analysis of Variance). If there is a significant difference, then it followed by the Duncan test at a confidence interval of 95% (α = 0.05).

RESULT AND DISCUSSION

Effects of Colchicine on Explant Growth and Shoot Formation

Crown explants cultured on MS media without colchicine formed green shoots (Fig. 1A). Meanwhile, addition of colchicine in concentration above 30 mg.L^{-1} inhibited shoot formation and growth (Fig. 1C, 1D, 1E).

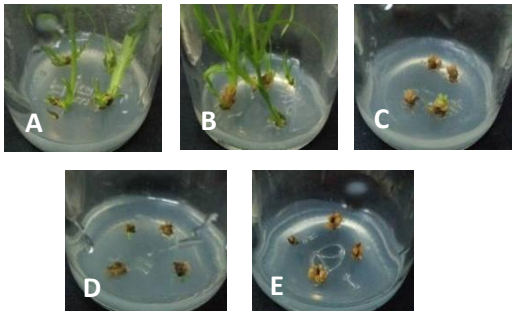


Figure 1. Effects of colchicine addition on the growth of explant crown for three weeks culture. (A). control (without colchicine), (B). 30 mg.L^{-1}, (C). 60 mg.L^{-1}, (D). 90 mg.L^{-1}, (E). 120 mg.L^{-1}

Colchicine's addition to the media affected the percentage of explants' survival, percentage of shoot formation, and the number of shoots. The higher concentration of colchicine on media caused the percentage of explant survival, percentage of shoot formation, and the number of shoot per explant was lower.

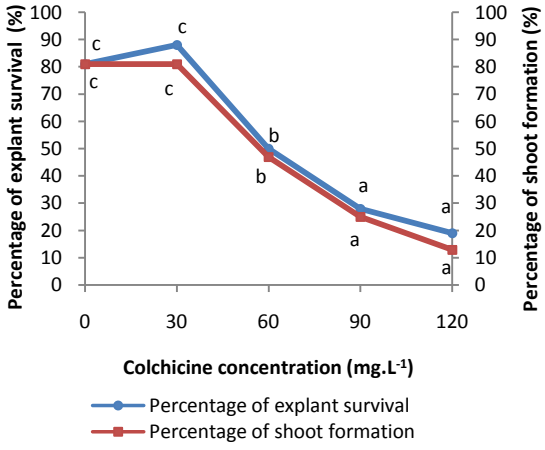


Figure 2. Effect of colchicine on percentage of explant survival and percentage of shoot formation (the same letter in the parameter showed no significance difference in Duncan Test α = 0.05).

In the media without colchicine, the percentage of explants survival and shoots formation was 81%. The addition of 60 mg.L^{-1} colchicine on media decreased the percentage of explants' survival and shoots formation to 50%

ISSN. 2087-2852
E-ISSN. 2338-1655

and 47%, respectively. Meanwhile, the percentages of explant survival and shoot formation with the addition of 120 mg.L^{-1} colchicine were only 19% and 13%. The highest percentage of explant survival (88%), as well as the highest percentage of shoots formation (81%), was recorded on MS media contains 30 mg.L^{-1} colchicine (Fig. 2).

The decrease in survival percentage of explants was caused by the toxic effects of colchicine. Colchicine, a highly poisonous alkaloid, originally extracted from *Colchicum autumnale*, is used in medicine, especially for the treatment of gout. Colchicine is a mutagenic chemical compound that can inhibit mitotic activity due to the binding of colchicine to tubulin and the nuclear spindle activity, thereby causing cell death [14]. Concentration above 0.1% of colchicine significantly decreased the percentage of survival explant and shoot regeneration in *Humuluslupulus* [15] and *Paulownia tomentosa* [16]. A high concentration of colchicine (above 0.4%) in *Pyrus communis* L. triggered cell death due to its toxic effect [17].

Total shoots and number of shoots per explant on the control media were 14 and 4 respectively on media. The addition of 60 mg.L^{-1} and 120 mg.L^{-1} colchicine on culture media reduced the number of shoot up to 50-85% (Fig. 3). The highest total shoot (17) and the number of shoot per explant (4) was observed on MS media contains 30 mg.L^{-1} colchicine.

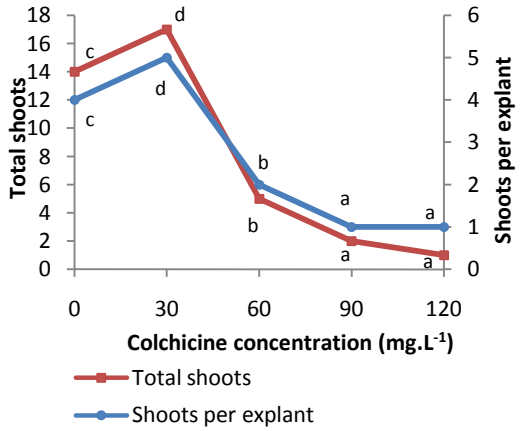


Figure 3. Effect of colchicine addition on media on the total shoots and number of shoots per explant (the same letter in the parameter showed no significance difference in Duncan Test α = 0.05).

The reduction of shoot formation in the treatment with colchicine has a negative side effect, due to the use of antimitotic agents for inducing polyploidy in plants. Extreme genetic redundancy and somatic instability after chromosome doubling affect cell proliferation, regeneration, and shoot elongation. The previous result also showed that the regeneration rate in *Rhododendron* was highly lethal and have aberrations like reduced growth rate. In addition to doubling meristematic cells, high concentration could reduce the overall vigor of the plant [18]. In Marigold, explants were reduced significantly from 87.83% on 0.001% colchicine to 27.26% on 0.05% colchicines. Colchicine inhibited the formation of spindle fibers, resulted in polyploid cells. Colchicine, as an antimitotic agent, binds to plant cell tubulin dimmers, causing depolymerization of microtubules, thus disrupting the cell cycle [19].

Plantlet Regeneration and Acclimatization

The shoots were able to form plantlets after colchicine treatment on MS media with 1 mg.L^{-1} NAA addition (Fig. 4A). The regenerated plantlets were dark green and large (Fig. 4B). Plantlet that successfully regenerated on media without colchicine was 23 plantlets. Addition of 30, 60, and 90 mg.L^{-1} colchicine on media result in 20, 5, and 19 plantlets respectively (Table 1); no plantlet obtained at 120 mg.L^{-1}.

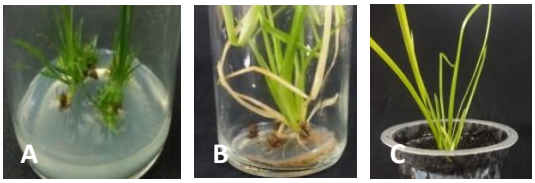


Figure 4. Plantlet regeneration and acclimatization from explant grown on media containing colchicines. (A). Shoot, (B). Plantlet, (C). Plant

Table 1. Effects of colchicine at different concentration on plantlet regeneration and acclimatization

Colchicine (mg.L^{-1})	Number of plantlets	Number of plants	Acclimatization success (%)
0	23	20	87
30	20	18	90
60	5	5	100
90	19	18	95
120	-	-	-

Four weeks after transferring the plantlet to the greenhouse, the plant of vetiver has a rather large, green leaves (Fig. 4C). The acclimatization success rate of the plantlet was 87% -100%. Plant obtained from control media were 20 plants. Whereas, the number of plants obtained from colchicine treatment at 30, 60, and 90 mg.L^{-1} were 18, 5, and 18 plants, respectively (Table 1).

Shoot survival in *Paphiopedilum villosum* decreased as the concentration of colchicine

ISSN. 2087-2852
E-ISSN. 2338-1655

increased [20]. Furthermore, there was no significant difference between diploid and higher ploidy for the length or width of leaves in *Hebe* sp. [21]. The acclimatization success rate of plantlet *Artemisia annua* reached up to 65.89%. Mixoploid clones (84%) and tetraploid clones (82.67%) from colchicine treatment showed a higher success rate than diploid clones (61.68%) [22]. The acclimatization of *Eriobotyra japonica* tetraploid plantlet showed slow growth and the resulted leaves were wider than diploid plants [23].

Ploidy Level Identification

Ploidy level of colchicine induced plants was determined by chromosomes counting in root-tips. Control media produced diploid plants (Fig. 5A). The addition of colchicine on media was able to induce mixoploidy, which is characterized by two or more types of ploidy in one individual plant. There was diploid (2n=2x=20), triploid (2n=3x=30) or diploid (2n=2x=20), tetraploid (2n=4x=40) (Fig. 5B).

The addition of colchicine to the culture medium induces cell polyploidy through chromosome doubling. Polyploidy is the condition of having more than two sets of chromosomes. Polyploidy can be achieved by chromosome doubling leads to changes in gene dosage that causes chromosomal rearrange-ments. Unreduced gametes transmit genetic diversity considered as effective alternatives for somatic chromosome doubling [24].

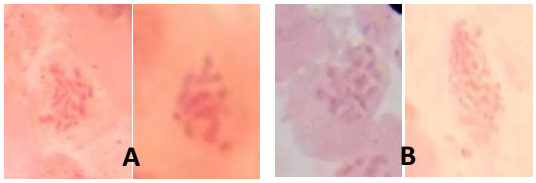


Fig 5. Vetiver cell chromosomes as a result of colchicine treatment by in vitro. (A) Diploid, (B) Mixoploid.

Addition of colchicine on media induced polyploidy of regenerated plantlet. Plant regenerated from explant treated colchicine of 30 mg.L^{-1} showed diploid level. Five mixoploids obtained from explant treated colchicine. One mixoploid (20%) obtained from the 60 mg.L^{-1} colchicine, and five mixoploids (62.5%) obtained by 90 mg.L^{-1} colchicine (Table 2).

Mixoploid is a condition in which the tissue composed of cells with different ploidy levels. Mixoploid shows that it was not all cells in the tissue exposed to colchicine compound changes in the amount chromosome. Mixoploidy is often associated with the occurrence of polyploidy, hybridization, chemical, and in the same case, it is genetically controlled [25].

Table 2. Effect of colchicine at different concentration on polyploid induction in *Vetiveria zizanioides*

Colchicine (mg.L^{-1})	Number of plant	Number of plants with observed roots	Plants ploidy (%)	
			Diploid	Mixoploid
0	20	5	100	0
30	18	7	100	0
60	5	5	80	20
90	18	8	37.5	62.5

Colchicine was a compound that used to induce polyploidy artificially. Colchicine as antimitotic compound, worked by duplicating the number of chromosomes, so that polyploid trait formed [23]. Colchicine induced polyploidy due to its ability to binds to tubulin proteins to inhibited polymerization of tubulin and no spindle threads, which plays a crucial role in cell division, so that chromatid separation couldn't happen [10]. Addition of 0.01 % of colchicine could induced polyploidy in *Bacopa monnieri* [26] and mixoploidy in *Physalis peruviana* L. [27]. Furthermore, addition of 0.1 % colchicine in *Trollius chinensis*is thought to be optimal to induce the polyploid plants [28].

CONCLUSION

Colchicine on media affected the shoot formation and growth of crown explants and induced the polyploidy of generated plantlets. The colchicine concentration above 30 mg.L^{-1} on media inhibited shoot formation and growth. However, 30 mg.L^{-1} colchicine can increase the number of the formed shoot.

The addition of 60 mg.L^{-1} and 90 mg.L^{-1} colchicine on media induced polyploidy in vetiver was 20% and 62.5%, respectively. Mixoploid vetiver plant consist of diploid (2n=2x=20) and triploid (2n=3x=30) cells or diploid (2n=2x=20) and tetraploid (2n=4x=40) cells.

ACKNOWLEDGMENTS

Part of this research was funded by the Higher Education Applied Research Project, Directorate of Research and Community Service, Directorate General of Research and Development Strengthening, Ministry of Research, Technology, and Higher Education.

REFERENCES

[1] Cedo, M.L.O., N. Ma Joahna, Licas, R.Z. Rocelie, C.D. Costancio. 2012. Enhanced Planlet Regeneration and In Vitro root

production in Vetiver (*Vetiveria zizaniodes* (L.) Nash). *Philpp. Agric. Sci.* 95. 344-351.

[2] Directorate General of Plantation. 2014. Statistik Perkebunan Indonesia/ Tree Crop Estate Statistics of Indonesia 2012-2014. Republic of Indonesia, Ministry of Agriculture. Directorate General of Plantation.

[3] Supriadin, J. 2017. Menjaga asa petani Garut kuasai minyak akar wangi dunia. Available at: http://regional.liputan6.com /read/3032176/menjaga-asa-petani-garut-kuasai-minyak-akar-wangi-dunia.

[4] Widoretno, W. 2016. In vitro induction and characterization of tetraploid Patchouli (*Pogostemon cablin* Benth.) plant. *Plant Cell Tiss. Org. Cult.* 125. 261-267.

[5] Lavania, U.C. 2003. Other uses, and utilization of vetiver: vetiver oil. *Central Institute of Medicinal and Aromatic Plants.* 38. 486-491.

[6] Evans, D.E., J.O.D Coleman, A. Kearns. 2003. Plant cell culture, 1st Ed. BIOS Scientific Publishers. United Kingdom.

[7] Xu, C., T. Tang, R. Chen, C. Liang, X. Liu, C. Wu, Y. Yang, D. Yang, H. Wu. 2013. A comparative study of bioactive secondary metabolite production in diploid and tetraploid *Echinacea purpurea* L. Moench. *Plant Cell Tiss. Org. Cult.* 116. 323-332.

[8] Dhooghe, E., K. van Laere, T. Eeckhaut, L. Leus, J. van Huylenbroeck. 2011. Mitotic chromosome doubling of plant tissues in vitro. *Plant Cell Tiss. Org. Cult.* 104(3).359-373.

[9] Dabkeviciene, G., V. Kemesyte, G. Statkeviciute, N. Lemeziene, G. Brazauskas. 2017. Autopolyploids in fodder grass breeding: induction and field performance. *Span. J. Agric. Res.* 15(4). 1-7

[10] Caperta, A.D., M. Delgado, F. Ressurreicao, A. Meister, R.N. Jones, W. Viegas, A. Houben. 2006. Colchicine-induced polyploidization depends on tubulin polymerization in c-metaphase cells. *Protoplasma.* 227. 147-153.

[11] Maikami, M., L. Kaveeta, S. Peyachoknagul, M. Nanakom. 2017. In vitro tetraploid induction of *Chrysopogon zizanioides* (L.) Roberty to improve salt tolerance. *Thai Agric. Res. J.* 35. 31-48.

[12] Ishigaki, G., T. Gondo, K. Suenaga, R. Akashi. 2009. Induction of tetraploid ruzigrass (*Brachiaria ruziziensis*) plants by colchicine treatment of in vitro multiple-shoot clumps and seedlings. *Jpn. Soc. Grassl. Sci.* 55. 164-170.

[13] Widoretno, W., A. Fauziah, A. Indriyani, E.P. Utomo. 2017. Clonal propagation of *Vetiveria zizanioides* L. through tissue culture technique. *Indonesian J. Essent. Oil.* 2(1). 38-44.

[14] Bennici, A., S. Silvia, M. Bruno. 2006. Morphogenic effect of colchicine in *Cichorium intybus* L. root explants cultured in vitro*. Caryologia.* 59(3). 284-290.

[15] Trojak, A, U. Skomra. 2013. Artificially induced polyploidization in *Humulus lupulus* L. and its effect on morphological and chemical traits. *Breed. Sci.* 63. 393-399

[16] Tang, Z.Q., D.L. Chen, Z.J. Song, Y.C. He, D.T. Cai. 2010. In vitro induction and identification of tetraploid plants of *Paulownia tomentosa*. *Plant Cell Tiss. Org. Cult.* 102. 213-220.

[17] Sun, Q., H. Sun, L. Li, R. L. Bell. 2009. In vitro colchicine-induced polyploid planlet production and regeneration from leaf explant of the diploid pear (*Pyrus communis* L.) cultivar, 'Fertility'. *J. Hortic. Sci. Biotech.* 84. 548-552.

[18] Ascogh, G.D., J. van Staden, J.E. Erwin. 2008. Effectiveness of colchicine and oryzalin at inducing polyploidy in *Watsonia lepida* N. E Brown. *Hort. Sci.* 43(7). 2248-2251.

[19] Qaoud, H.A., J.Y.S. Munqez. 2014. The effect of colchicine on adventitious shoot regeneration from cultured leaf explants of *Petunia hybrida*. *Br. Biotechnol. J.* 4(5).531-540.

[20] Huy, N.P., D.T.T. Tam, V.Q. Luan, H.T. Tung, V.T. Hien, H.T.M. Ngan, P.N. Duy, D.T. Nhut. 2019. In vitro polyploidy induction of *Paphiopedilum villosum* using colchicine. *Sci. Hortic.* 252. 283-290.

[21] Gallone, A., A. Hunter, G.C. Douglas. 2014. Polyploid induction in vitro using colchicine and oryzalin on Hebe 'Oratia Beauty': Production and characterization of the vegetative traits. *Sci. Hortic.*179. 50-66.

[22] Rahman, W., E. Al Hafiizh, T.M. Ermayanti, D.E. Rantau, A.A Lelono. 2017. Acclimatization and agronomic performance of polyploids clones of *Artemis annua* L. *Jurnal Biologi Indonesia.* 13(1).33-41.

[23] Blasco, M., M.L. Badenes, M. del M. Naval. 2014. Colchicine-induced polyploidy in loquat (*Eriobotrya japonica* (Thunb.) Lindl.). *Plant Cell Tiss. Org. Cult.* 120. 453-461.

[24] Manzoor, A. Tuqeer, A.B. Muhammad, A.H. Ishfaq, S. Christian. 2019. Review studies on colchicine induced chromosome doubling for enhancement of quality traits in ornamental plants. *Plants.* 8(194). 1-6.
[25] Ranjbar, M., K. Roya, N. Samineh. 2011. Diploid-tetraploid mixoploidy in a new species of *Astragalus* (Fabaceae) from Iran. *Ann. Bot. Fennici*. 48. 343-351.
[26] Escandon, A.S., J.C Hagiwara, L.M. Alderete. 2006. A new variety of *Bacopa monnieri* obtained by in vitro polyploidization. *Electron. J. Biotechnol.* 9(3). 181-186.
[27] Garcia-Arias, F., E. Sanchez-Betancourt, V. Nunez. 2018. Fertility recovery of anther-derived haploid plants in Cape gooseberry (*Physalis peruviana* L.). *Agronomia Colombiana.* 36(3). 201-209.
[28] Zhang, Q., F. Zhang, B. Li, L. Zhang, H. Shi. 2016. Production of tetraploid plants of *Trollius chinensis* Bunge induced by colchicine. *Czech J. Genet. Plant Breed.* 52(1). 34-38.

ISSN. 2087-2852
E-ISSN. 2338-1655

Assessment of Water Quality Using Benthic Macroinvertebrate along Sumber Maron River, District of Gondanglegi Kulon, East Java-Malang, Indonesia

Faiza Tawati[1,2], Yenny Risjani[3], Sasmito Djati [4], Bagio Yanuwiadi[4], Amin Setyo Leksono[4]

[1]Doctoral Program of Environmental Science, Postgraduate School, University of Brawijaya, Malang, Indonesia
[2]Higher Institute for Comprehensive Profession, Gharabuli, Libya
[3]Faculty of Fisheries and Marine Sciences, University of Brawijaya, Malang, Indonesia
[4]Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia

Abstract

Human activities such as agriculture, swimming, livestock, public washing, and the use of latrines around the river are considered as major causes of damage to the river ecosystem, affecting the growth of Macroinvertebrate, and thus assessing the condition of the river. This study aimed to study the composition of Macroinvertebrate and the quality of water health in Sumber Maron River, in the village of Gondanglegi. This research was conducted from March to April 2018. The method used in this research was the quantitative method using primary data. Research stations were identified by considering land-use in nine regions. The sampling technique of total macroinvertebrates was the kicking technique using the manual mesh with a 500 µm applied to the submerged solid substrates. The data were analyzed using a BMWP Index and interpreted by calculating the Average Scores Per Taxon (ASPT). The result of this research represented that there were 20 taxa of macroinvertebrates, which consisted of It belongs to 8 orders (Trichoptera, Coleoptera, Diptera, Tricladida, Decapoda, Odonata, Ephemeroptera, Plecoptera) and four class (Oligochaeta, Hirudinea, Gastropoda, Crustacea). Generally, the lowest taxa groups found in the station A1 (3 taxa), which were the agriculture and toilet (recreation area). Meanwhile, the highest taxa groups were found in station B3, which was the recreation area, by a total of 14 taxa. Macroinvertebrate with the lowest abundance was Gerridae at station A3, which respectively had the number of 1 ind.$5m^{-2}$. Macroinvertebrate with the highest abundance was Baetidae at the station B3, which is the recreation area, by the number of 549 ind.$5m^{-2}$. The result of modified BMWP – ASPT analysis showed a value of 3.3 – 5.75, which indicated that the condition of Sumber Maron River was ranging from bad to excellent category. The bad water condition was found in the agriculture and toilet (recreation area), whereas the excellent water condition was found in the swimming pool (recreation area). Canonical Correspondence Analysis (CCA) revealed that environmental variables were significant for an explanation of the variance in the family.

Keywords: BMWP-ASPT, Macroinvertebrate, River.

INTRODUCTION

The river is open water that is easy to be influenced by the surrounding area, either naturally or from human activities. The river and its streams are an integral part of the watershed area. There are two important factors that can affect the river ecosystem, namely the constantly flowing water as well as the input of organic matter from the surrounding area. Sumber Maron River is an area that attracts visitors and tourists. It is one of the environmental tourism located in the south of Malang, where there are natural animals and plants.

Sumber Maron consists of three areas, the upstream, central, and estuary, each of which has different characteristics. The river water is used for household purposes, daily washing, cooking, bathing, and personal cleaning. The river is also used for swimming and recreation. Although Sumber Maron is an area of springs that must be protected and away from business activities or other activities that can harm the environment with the waste they produce, but along this attraction, there are many food stalls scattered with a variety of menus. While dining in this tourist area, especially in canned food, people may not be aware of dumping surrounding wastes and damaging.

Sumber Maron River contaminated in some areas due to waste from human activities. In addition, pollution comes from domestic, agricultural, and industrial waste. The most obvious result is an increase in the level of organic matter in water that is likely to decrease [1].

* Correspondence address:
Faiza Tawati
E-mail : fzaya2004@gmail.com
Address : Postgraduate School, University of Brawijaya, MT Haryono 169, Malang 65145.

ISSN. 2087-2852
E-ISSN. 2338-1655

Pollutants are organic and inorganic substances derived from human activities around watersheds. Thus, water quality is affected. Furthermore, changes in water quality will affect the status and composition of the total living invertebrates of the river ecosystem. Macroinvertebrates are selected with their relatively stable characteristics as a biological indicator of water quality degradation due to the increasing activities human factors around watersheds. The community of these organisms has a rapid and consistent response to physical and chemical changes in water quality, thus can describe long-term environmental conditions [2].

In addition, the bioaccumulation study is not only used to detect environmental changes but can also provide river resource management options and recommendations for bioprospecting studies on species that can be selected as test animals [3]. The author, therefore, conducted research on bio-assessment of the river using the BMP index to study the overall composition of invertebrates and determine the health status of the Sumber Maron River in Village of Kepanjen, East Java.

MATERIAL AND METHOD

This study used descriptive qualitative method. A qualitative method was used to explore a problem that occurred. The process of this method includes the submission of questions and procedures, as well as collecting specific data from the research object. A qualitative method was used to examine the natural objects (opposite of the experimental method). The natural object itself is the object that is happened and characterized naturally without any intervention or manipulation by the researcher [4]. In this study, a survey was conducted directly for the sampling of macro-invertebrates and river water at the predetermined research stations.

Sumber Maron River is located in the administrative area of Karangsuko Village, Malang regency. Sumber Maron is one of the river areas near the springs, which had been set as a tourism destination in Malang regency since 2007. The management of the tourism destination set the protection of a minimum of 200 meters from the spring. The elevation of the area is 300 and 337.5 meters above sea level. The temperatures range 17.25°C and 30.7°C with the precipitation on the average of 1761 mm per year and the rainy duration of 84.85 days per year [5].

Data Collection

The method of data collection in this research was observation, which is elaboorated in the selection, alteration, recording and coding of a series of behaviors and circumstances regarding the in-situ organisms in accordance with empirical goals [6]. The observation was obtained by observing research locations along the upstream area of Sumber Maron River in Gondanglegi Village to collect macroinvertebrate and samples directly.

Sample was then assessed using modified index analysis of Biological Monitoring Working Party (BMWP). Furthermore, the observation of macroinvertebrate and measurement of water parameters were performed in the laboratory. The determination of sampling stations was based on the map used in the surrounding area of upped Sumber Maron River in Gondanglegi Village (Fig. 1).

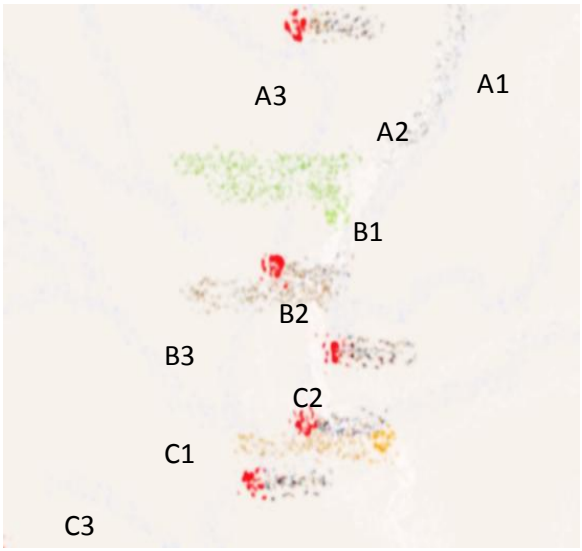


Figure 1. Macroinvertebrates and Water Sampling Stations
Description:
Upper Stream: A1, A2, A3
Middle Stream: B1, B2, B3
Downstream: C1, C2, C3

Sampling Technique

The macroinvertebrate sampling technique used the kicking method. Macroinvertebrate sampling tools and procedures were conducted in shallow waters allowing researchers to directly conduct direct sampling in rivers by using a wading suit, along 10 m in the riffle [1]. Brabander way of macroinvertebrate sampling was performed in an

ISSN. 2087-2852
E-ISSN. 2338-1655

area of 10 to 20 m using a hand net, a mesh size of 500 µm [7]. The sample was collected for 10 meters for each research station and was obtained on a shallow area of the river with a hard substrate. The preservation of samples was using containers with alcohol 96%. Macroinvertebrates were identified to the family level.

Data Analysis

Analysis of the health status of Sumber Maron River is done after the identification of macroinvertebrate by calculating the composition and relative density of macroinvertebrate from each research station. Relative density is the percentage of individuals numbers per family divided to the total of individuals from the entire family of macroinvertebrate.

Macroinvertebrates are assessed using the BMWP (Biological Monitoring Working Party) index and calculated using the ASPT (Average Score Per Taxon). The results were then categorized according to the ASPT value. ASPT was calculated by dividing the score of the number of 1 family with the total number of the entire families from 1 sample [8]. ASPT score is categorized into six groups of interpretation (Table 1). Canonical Correspondence Analysis (CCA) with UPGMA cluster analysis also applied to reveal which environmental variables were significant to explain the variance in family abundance patterns.

$$\text{ASPT} = \frac{\text{Total score of BMWP Index}}{\text{The number of the Family found}}$$

Table 1. ASPT score category

ASPT Score	Water Quality
>6	Excellent
5.5 – 6.0	Very Good
5.0 – 5.5	Good
4.5 – 5.0	Moderate
4.0 – 4.5	Moderate – Poor
>4.0	Poor

Source: Galbrand *et al.* [8].

RESULT AND DISCUSSION

Environmental Parameters

Water quality of Sumbermaron River at Gondanglegi Kulon Village, Malang, East Java described as follows. Substrates of the river base vary from large rocks, small stones, gravel, and sand. Physical and chemical properties are important for knowing water quality, as well as for supporting and assisting the life of macroinvertebrate. The results can be seen in Table 4. Temperatures ranged from 23.7-24°C and are considered a good range for the aquatic organism (optimum 20-30°C) [9]. The pH level of all stations is 7 and is still ideal for the ecosystem [10]. The hardness level ranged from 192-200 mg.L^{-1} and classified as hard water [9]. BOD_5 level ranged from 3.8–14.5 mg.L^{-1} classified as polluted for natural waters [11], and COD level ranged from 12.24–50.54 mg.L^{-1}.

Table 2. Physical and chemical indicators of each station

Prop.	Station (A)	Station (B)	Station (C)	WHO
°C	23.97±0.153	23.9±0.264	23.8±0.200	15
pH	7.47±0.208	7.63±0.115	8.4±0.96	6.5-8.5
BOD	8.51±4.34	8.20±5.59	6.28±3.74	5
COD	30.12±19.28	29.15±19.84	23.85±14.19	0.2
HD	200.67±5.03	196.67±3.06	194.0±5.29	150

Notes: Prop. = physiscal and chemical properties, °C = temperature, HD = hardness.

Macroinvertebrate Composition

Observations of macroinvertebrate communities were conducted in the rainy and dry season. Parameters calculated in this study consisted of the abundance, relative density, richness, diversity, and composition. The results of observations of the macroinvertebrate composition were shown in Table 3 and Figure 2. There were 5389 individuals of macro-invertebrates collected in all stations. The abundance of the macro-invertebrates collected in the rainy season (474.11 ±135.55) was higher than that in the dry season (124.67±53.52) (Table 3). All dominant families' abundance had shown the same trend.

Table 3. Mean of abundance of Macroinvertebrata collected from all stations in rainy and dry seasons

Family	Season	
	Rainy	Dry
Baetidae	230.11 ± 69.01	46.67 ± 32.99
Hydrobiidae	89.89 ± 43.66	30.78 ± 9.41
Chironomidae	43.44 ± 20.49	22.33 ± 10.56
Hydropsychidae	33.22 ± 13.83	8.67 ± 6.70
Heptagenidae	33.89 ± 20.35	5.22 ± 3.72
Tipulidae	20.11 ± 18.25	0.22 ± 0.22
Atydae	4.67 ± 2.59	2.78 ± 1.69
Grapsidae	4.89 ± 2.41	0.89 ± 0.56
Elmidae	3.56 ± 1.36	1.56 ± 0.78
Rhyachophilidae	3.89 ± 1.79	0
Miscellenies	8.44 ± 2.15	7.56 ± 2.90
Total abundance	474.11 ± 135.55	124.67 ± 53.52

Observations and identification conducted during the study showed that macroinvertebrates found in the Sumber Maron River consisted of 18 families in the rainy season and 16 families in the

ISSN. 2087-2852
E-ISSN. 2338-1655

dry season. It belongs to 8 orders (Trichoptera, Coleoptera, Diptera, Tricladida, Decapoda, Odonata, Ephemeroptera, Plecoptera) and fourclass (Oligochaeta, Hirudinea, Gastropoda, Crustacea).

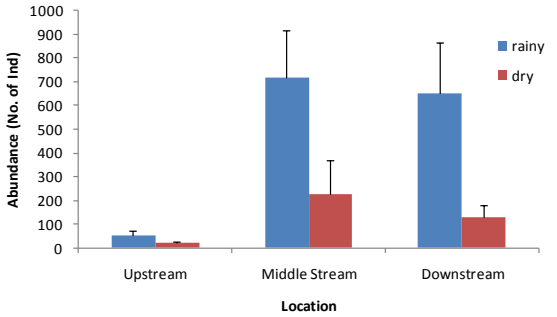


Figure 2. The abundance of macro-invertebrates in all locations both in rainy and dry seasons

The lowest mean of individuals was found in upstream both in the rainy season (53.67±19.33 individual) and in the dry season (19.61±4.04 individual). Meanwhile, the highest was found in the middle stream both in the rainy season (715.67±228 individual) and in the dry season (202.9±143.09 individual). The stations in upstream had the lowest abundance due to substrate that is dominated by sand and mud. Some species live among vegetations, and the others live in the mud. It is because the pillars of this station differed from sand and gravel to rock. Odum explained that flat and gravel environments are good environments for large invertebrates and an environment suitable for invertebrate growth [12].

The lowest mean of families was found in upstream, both in the rainy season (5.67±1.76 families) and in the dry season (4.66 ± 0.88). That station had the lowest number of taxa due to the substrate that is dominated by sand and mud. Some species live among vegetations, and the others live in the mud.

The highest number of families was found in the middle stream with the mean of 12.33 ±1.2 families in rainy season dan 10.67±1.67 families in the dry season. Those were including Chironomidae, Tipulidae, Baetidae, Caenidae, Hydropsychidae, Rhyacophilidae, Grapsidae, Atydae, Hydrobiidae, Valvatidae, Perlidae, Planaridae, Elmiidae, and Lumbriculidae. Substrates of this station were varied from sand, gravel to rocks. Flat stone and gravel are good environments for macroinvertebrates [12].

The lowest diversity was found in upstream, both in the rainy season (1.18±0.18) and in the dry season (1.12±0.13) Figure 3. The highest diversity was found in the middle stream with the mean of H' index was 1.42±0.05 in dry season Figure 4. Based on the score, the diversities of Macro-invertebrate were considered fair condition.

Statistical analysis of variance showed that the taxa richness (F=12.94; P<0.001) of macro-invertebrates were significantly higher in the middle stream, while there were no significant differences of the abundance and diversity between seasons and among locations. Several groups showed variation in the abundance between seasons and among locations. Baetidae, Hydrobiidae, and Rhyachophilidae responded to the seasons. Meanwhile, Baetidae, Hydrobiidae, Hydropsychidae, Atydae, and Rhyachophilidae responded to the locations. The interaction effect of season and location was significant to Hydrobiidae and Rhyachophilidae (Table 4). However, many families were not significantly different between season and among stations.

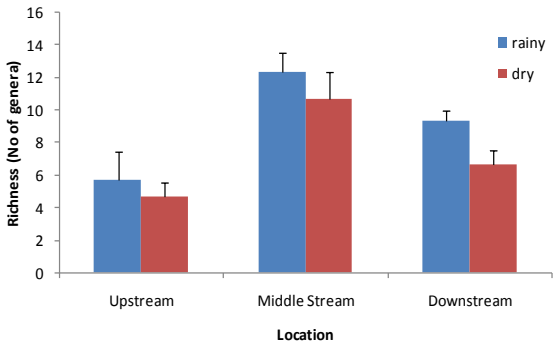


Figure 3. The taxa richness (number of families) of macro-invertebrates in all locations both in rainy and dry seasons

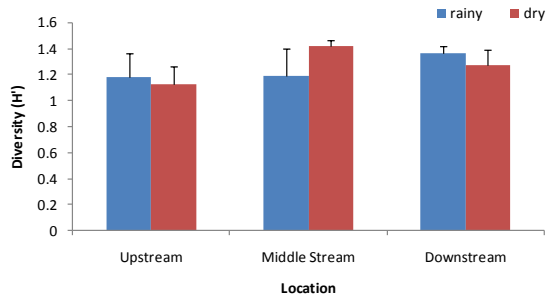


Figure 4. The diversity of macroincvertebrates in all locations both in rainy and dry seasons

ISSN. 2087-2852
E-ISSN. 2338-1655

Tabel.4. Summary of *F* values followed by degree of significance using ANOVA of the abundance, taxa richness and diversity of several Macro-invertebrate (Benthos) families

Group	Season (S)	Location (L)	S*L
Baetidae	13.22**	9.31**	3.08
Hydrobiidae	4.95*	10.77**	5.84*
Chironomidae	0.76	0.84	0.41
Hydropsychidae	4.00	5.11*	1.45
Heptagenidae	2.00	1.23	1.12
Tipulidae	1.21	1.08	1.03
Atydae	0.54	5.19*	0.37
Grapsidae	2.24	0.24	0.63
Elmidae	1.27	0.25	0.05
Rhyachophilidae	36.03***	27.56***	27.56***
Miscellenies	0.09	3.35	1.81
Abundance	9.95	5.82	2.03
Taxa richness	3.05	12.94***	0.23
Diversity	0.06	0.90	0.81

Among the families, Baetidae, Hydrobiidae, Chironomidae, and Hydropsychidae were dominant in all stations both in rainy and dry seasons (Figure 5). These dominant families classified as a combination of moderate polluted groups, e.g. Hydropsychidae, Baetidae, and Gastropoda: Hydrobiidae, and heavily polluted, e.g. Chironomidae. Baetidae can be used as a bioindicator of mild pollution originating from organic materials. Based on the description of INP, it was concluded that the farther the spring was, the lower the organic material pollution level, or the better water quality of the channel, as indicated by the increase in the abundance of varieties in Baetidae.

Quingley [13] states that Chironomidae can be found in all types of water and prefer to live in mud. Chironomidae is tolerant of polluted water. Chironomidae can also be used as an indicator that the majority of the river's land is mud.

Diptera is a type of large invertebrate that loves to live in the mud substrate [14]. This means that the discovery of Chironomidae in large quantities means very suitable for gravel, sand, and mud. The Hirudinea group can withstand low oxygen levels and high levels of water pollutants [15]. It means the river polluted with high organic pollutants due to Planaridae found in it.

The result of the UPGMA cluster analysis showed that micro-invertebrate was assemblage according to season except in upstream. Macroinvertebrate compositions in rainy seasons between the middle stream and downstream branched at 56.1%. Macroinvertebrate compositions in dry seasons between the middle stream and downstream branched at 51.5%. Branching of the macroinvertebrate compositions in upstream between rainy and dry seasons occurred at 49.3%. All compositions from two seasons and three locations had less than 20% similarity (Fig. 6).

The level of similarity between communities in the range of 40-60 is classified medium while under 40% is classified as low. It shows that macro-invertebrate spread is relatively uneven. This situation may because macroinvertebrates classified as sessile animals with relatively limited movement [16].

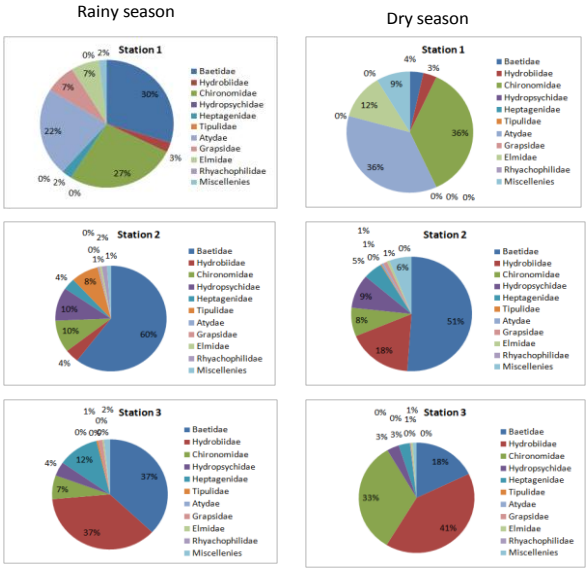


Figure 5. The number of macroinvertebrates families for each station in Sumber Maron River in rainy and dry seasons

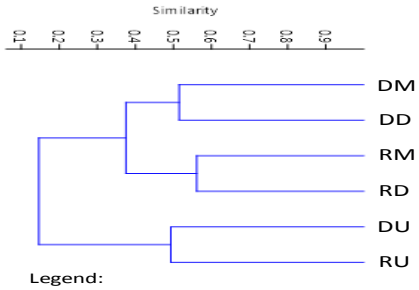


Figure 6. Community compositions between macro-invertebrate between rainy season and dry season and among the locations

ISSN. 2087-2852
E-ISSN. 2338-1655

Canonical Correspondence Analysis (CCA) revealed that environmental variables were significant for the explanation of the variance in family abundance patterns (Fig. 7). The sum of the first two canonical eigenvalues was 0.45. The first axis explained 46.4% of the family-environment relations, while the second explained 37.6% of the family-environment relations. All environmental factors had no significant effect on the family-environment ($P > 0.05$). It means the distribution of family abundance between season and among the sites was not influenced by environmental factors.

Although some families largely responded to the season, the environmental factor did not shape the formation in general. Pollution of the environment is one of the most important problems and challenges facing the world. Rivers are exposed to pollution as a result of the dumping of wastes loaded with biodegradable organic matter such as the remains of dead foods and animals and pesticide residues. Empty containers or toxic and hazardous chemicals containing pathogens found in rivers and valleys, the community is exposed to many diseases. It reduces the quality of freshwater for uses and becomes a barrier to human health, agriculture, and industry. Water pollution leads to a shortage and scarcity of environmental resources and health and social problems negatively affect the economic performance that will threaten food security.

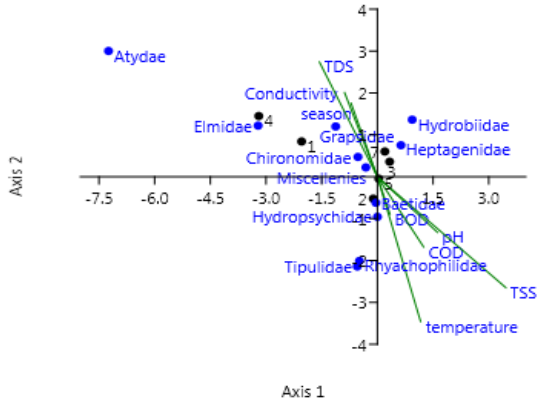


Figure 7. Analysis of CCA of macroinvertebrate family group in response to environmental variables

Analysis of River Health Status

The analysis of the health status of Sumber Maron River in Gondanglegi Village was obtained using the Biological Monitoring Working Party (BMWP) index and the calculation of ASPT. The result demonstrated a score of 3.33-5.75. It indicates that the water quality of the Sumber Maron River ranged from poor to very good quality. Based on the analysis by using the modification of the BMWP index, the result of the classification of macroinvertebrates in Sumber Maron River, Gondanglegi Village, Kepanjen obtained from the grouping of nine observation stations as in Table 4.

Table 4. Classification of macroinvertebrata in Sumber Maron River of nine stations

No.	Station	ASPT	Water Quality Status
1	Station A1	3.33	poor
2	Station A2	4.2	moderate-poor
3	Station A3	2.66	poor
4	Station B1	4.55	moderate
5	Station B2	4.71	moderate
6	Station B3	4.88	moderate
7	Station C1	4.8	moderate
8	Station C2	5.75	very good
9	Station C3	4.85	moderate

The result of BMWP modification analysis that belongs to very good waters is station C2, which has ASPT value 5.75. The high value of ASPT at the station is due to Heptagenidae, Caenidae, and Perlidae. Water quality at station C2 is the current velocity as high as fast, high dissolved oxygen, type of large rock substrate, gravel, and sand. Plecoptera species are present among the litter, algae, or under rocks in flowing waters, usually found only in waters with high oxygen content, never present in polluted waters, to be used as biological indicators. Therefore, the presence of Trichoptera and Plecoptera larvae is used as an indicator that the aquatic environment is good [17,18].

Although at station C2 is still very good waters, it has obtained a type of macroinvertebrate that has a low score, namely Chironomidae. A river dam has been created at this station for recreation purposes. It can contribute to the level of hardness derived from the use of soap.

The results of BMWP modification analysis that are classified as moderate water quality are station A2, B1, B2, B3, C1, and C3, which has an ASPT value ranging from 4.2 to 4.88. Macroinvertebrates with the lowest scores were found at these stations because it was used as tourist attractions (pools). Tourists often swim and influence the substrate changes; the substrate is strongly related to life for macroinvertebrates [19]. If there is a rainy season,

ISSN. 2087-2852
E-ISSN. 2338-1655

then the land on the plantation land will fall into the river that will cause changes to the bottom of the river basin into the mud, and mud is a good habitat for the Diptera class. Diptera is a type of macroinvertebrate that prefers to live on mud substrate, as well as most Chironomidae larvae found in a shallow pond, lake, and calm river [15].

The result of BMWP modification analysis, which classified as very bad (poor quality waters) are A1 and A3 stations, with ASPT value ranged between 3.33 and 2.66. Macroinvertebrates with the lowest scores have been found in this station. It is due to the discovery of Lumbriculidae type macroinvertebrates. Also, these stations have been used as tourist attractions (pools), where tourists often swim and influence substrate changes, where the substrate is strongly related to life for macroinvertebrates.

CONCLUSION

The chemical, physical, and biological properties of water quality in the Sumber Maron River were obtained as follows. The temperature ranged from 23.6-24.2°C, the pH of the water was 7-9, hardness ranged between 190-206 $mg.L^{-1}$, BOD ranged from 3.8-14.5 $mg.L^{-1}$, COD ranges from 10.31-50.54 $mg.L^{-1}$, and all results are within WHO standards.

The abundance of macroinvertebrates collected in the rainy season was higher than that in the dry season and all the dominant families' abundance showed the same trend. The macroinvertebrates found in the Sumber Maron River consisted of 18 families in the rainy season and 16 families in the dry season, which belong to 8 orders.

The result demonstrated a score of 3.33-5.75. It indicates that the water quality of the Sumber Maron River ranged from poor to very good quality.

REFERENCES

[1] Sudaryanti, S., Kusriani, Wijarni, M. Mahmudi, and E.Y. Herawati. 2003. Profile of student research results in 1998-2002. Program Study of Water Resource Management. Faculty of Fisheries and Marine Science, University of Brawijaya. Malang.

[2] Mulyanto. 1995. Makrobenthos sebagai indikator biologi perubahan kualitas air di Sungai Amprong Malang. Faculty of Fisheries and Marine Science, University of Brawijaya. Malang.

[3] Sudaryanti, S. 2002. Biodiversity of aquatic ecosystems for monitoring environmental conditions. Training of Land Management and Conservation. Center for Environmental Research, University of Brawijaya – BAPEDAL of East Java – Australia BEJIS Project. January 2002.

[4] Cantonati, M., K. Ortler. 1998. Using spring biota of pristine mountain areas for long-term monitoring. In: Hydrology, Water resources and Ecology in headwaters. Proceedings of the Head-Water '98 Conference. Meran/ Merano, Italy. IAHS Publ. 248. 379-385.

[5] Pandulu, G.D. 2015. Penataan ruang kawasan Sumber Air Jeruk dan Maron Kabupaten Malang. *Jurnal Reka Buana*.1(1). 10-18.

[6] Hasan, M.I. 2002. Pokok-pokok materi metodologi penelitian dan aplikasinya, 1st Ed. Ghalia Indonesia. Jakarta.

[7] Sudaryanti, S., Marsoedi. 1995. Pendekatan biologis untuk menduga kualitas air Sungai Brantas Jawa Timur. Faculty of Fisheries and Marine Science, University of Brawijaya. Malang. *Buletin Perikanan*. 52.

[8] Galbrand, C., I.G. Lemieux, A.E. Ghaly, R. Cote, M. Verma. 2007. Assessment of constructed wetland biological integrity using aquatic macroinvertebrate. *Online J. Biolo. Sci.* 7(2). 52-65.

[9] Effendi, H. 2003. Telaah kualitas air, 5th Ed. Kanisius. Yogyakarta.

[10] Barus, T. A. 2002. Pengantar limnologi. Department of Biology, Faculty of Mathematics and Sciences, University of North Sumatra. Medan.

[11] Republic of Indonesia. 2001. Government Regulation No. 82 year 2001 about water quality standards. Republic of Indonesia. Jakarta.

[12] Odum, E.P. 1993. Fundamentals of ecology, 3rd Ed. Samingan, T. (transl). Gadjah Mada University Press. Yogyakarta.

[13] Quigley, M. 1977. Invertebrates of streams and rivers: a key to identification. Edward Arnold. Nene College. Northampton.

[14] Hawking, F.J. Smith 1997. Colour guide to invertebrates of Australian inland water. Identification Guide Freshwater Ecology.

ISSN. 2087-2852
E-ISSN. 2338-1655

Murray Darling Freshwater Research Centre. Albury NSW.

[15] Gooderham, J., E. Tsyrlin. 2002. Waterbug book: a guide to the freshwater macro-invertebrates of temperate Australia. DOI: 10.1071/9780643090026.

[16] Ormerod, S.J., R.W. Edwards. 1987. The ordination and classification of macro-invertebrate assemblages in the catchments of the River Wye in relation to environmental factors. *Freshwat. Biol.* 17. 533-546.

[17] Awuy, N.H.S., S. Sudaryanti. 2003. Pemberdayaan komunitas makrozoobenthos untuk Pengelolaan Sungai Lang-lang Kecamatan Singosari, Kabupaten Malang, Jawa Timur. National Seminar Faculty of Mathematics and Science. Sepuluh Nopember Institute of Technology, Surabaya.

[18] Patang, F., A. Soegianto, S. Hariyanto. 2018. Benthic macroinvertebrates diversity as bioindicator of water quality of some rivers in East Kalimantan, Indonesia. *Int. J. Ecol.* Article ID 5129421, 11.

[19] Boyero, L. 2003. The quantification of local substrate heterogeneity in streams and its significance for macroinvertebrate assemblagess. *Hydrobiologia*. 499. 161-168.

ISSN. 2087-2852
E-ISSN. 2338-1655

Effect of Benzyl Adenine Concentration on Callus Induction of Geranium Plants (*Pelargonium graveolens* L'Her) from Petiole and Leaf Explants

Moch. Faizul Huda, Serafinah Indriyani, Wahyu Widoretno*

Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia

Abstract

Geranium plant (*Pelargonium graveolens* L'Her) is one of the geranium oil-producing plants that has many benefits. Callus culture is a technique that can be used to plant multiplication and increase production of secondary metabolites. This study aims to determine the effect of the concentration of Benzyl Adenine on the formation of geranium callus from petiole and leaf explants. Callus induction was carried out by culturing petiole and leaf explants on MS medium + 0.1 mg.L^{-1} NAA + Benzyl Adenine (0; 0.5; 1; 1.5 and 2 mg.L^{-1}). Callus morphological parameters, percentage of callus formation, and time of first callus formation were observed. The formation of geranium callus influenced by the explant type and the concentration of Benzyl Adenine. In the 2nd week, the geranium callus was initiated, light green colored with a compact callus texture. At 4th week, the percentage of callus formation containing NAA 0.1 mg.L^{-1} of petiole and leaf explants was 20% and 8%, whereas the percentage of callus formation on medium containing 0.1 mg.L^{-1} NAA combined with 0.5-2 mg.L^{-1} Benzyl Adenine of petiole and leaf explants was 52-80% and 24-52%. The best percentage of callus formation was found on the culture medium containing 1 mg.L^{-1} Benzyl Adenine, equaled 80% of petiole explants, and 52% of leaf explants.

Keywords: BA, Callus, Leaf, Petiole, *Pelargonium graveolens* L'Her.

INTRODUCTION

Pelargonium graveolens L'Her or known by the name Geranium plant is one of the geranium essential oil-producing plants that have many benefits such as cosmetics, perfume and can overcome several health problems [1-2]. The high demand for geranium oil has not been well fulfilled by total production. Global geranium oil production is estimated at only 250-300 tons per year, while demand for geranium oil is more than 800 tons per year [3]. Based on this there is an opportunity to increase geranium oil production through callus culture techniques.

Callus culture techniques in addition to plant propagation techniques are also one of the techniques for the production of secondary metabolites [4]. Callus culture techniques has several advantages such as controlled environmental factors so that it is not influenced by climate, season, pests and plant diseases and can produce secondary metabolites that are more consistent in a shorter period of time [5].

Growth and formation of callus in culture was influenced by plant growth regulators (PGR) auxin and cytokinin. The use of PGR alone or in combination with the right concentration can induce and increase callus growth so that better results are obtained. The use of a combination of PGR auxin and cytokinin greatly influences the determination of the type of morphogenesis. The balance of hormones in cells against auxiliary PGR auxin and cytokinin determines the differentiation process [6-7]. The combination of auxins and cytokinins properly stimulates cell division so that it can induce callus formation [8].

Callus induction is strongly influenced by the type and concentration of plant growth regulators. NAA and BA are types of auxins and cytokinins commonly used for callus induction. In *Astragalus nezakate* plants, the response of callus formation and bud regeneration on MS medium with the addition of NAA and BA was more effective than the addition of other plant growth regulators [9]. The use of 0.05 mg.L^{-1} NAA and 5 mg.L^{-1} BA could increased the frequency of callus formation in *Catalpa bungei* [10]. *Arthemisia absinthium* callus induction on MS medium with the addition of BA combined with NAA, 2,4-D or IBA is also able to form callus up to 100% [11].

The use of different types of explants can also provide a different callus growth response [12]. The use of appropriate explants in each species is a major factor in the success of callus culture. Callus formation from *Eurycoma longifolia* Jack explants was faster than leaf explants [13]. While the use of *Catalpa bungei* stem explants is less responsive in forming callus compared to leaf explants [10].

*Correspondence address:
Wahyu Widoretno
Email : wahyu_widoretno@yahoo.com
Address : Dept. Biology, University of Brawijya, Veteran Street, Malang, Indonesia. 65145

ISSN. 2087-2852
E-ISSN. 2338-1655

The results of these studies, it appears that the response of callus formation depends on plant genotype, type of explant and formulation of the medium used. This study aims to determine the effect of Benzyl Adenine concentration on callus formation of geranium plants (*Pelargonium graveolens* L'Her.) from petiole and leaf explants.

MATERIALS AND METHODS

Petiole and leaf explants of geranium plant (*Pelargonium graveolens* L'Her) were used for callus induction. The explants surface was sterilized using a 20% commercial whitening solution (containing 5.25% NaClO) for 5 minutes and rinsed by using sterile distilled water twice each for 5 minutes. Sterile explants were cultured on MS medium with the addition of plant growth regulators, namely NAA (0.1 mg.L^{-1}) combined with several concentrations of plant growth regulators BA (0; 0.5; 1; 1.5 and 2 mg.L^{-1}). Each treatment was repeated 5 times (bottle), each bottle was cultured with 5 explants. The culture was incubated at room temperature (25 ± 1)°C and 600 Lux light.

The effect of each combination treatment of plant growth regulators on petiole and leaf explant was determined by observing callus morphology and percentage of callus formation, including time of first callus formation. Quantitative data were analyzed using ANOVA, and the averages compared using the Duncan multiple range tests (DMRT) at a significance level of 5% ($P < 0.05$).

RESULTS AND DISCUSSION

Geranium callus can be induced from petiole and leaf explants cultured on MS medium with the addition of plant growth regulator Benzyl Adenine (BA) combined with NAA. Formation of the geranium callus was initiated in the 2nd week after induction. The formed geranium callus was light green with a compact callus texture first visible on the edge of the explant cut, followed by the entire explant surface (Fig. 1).

Induction of geranium callus influenced by the type of explant and BA concentration, both factors have a significant effect on callus formation and growth. At 4th week after induction, the percentage of callus formation from petiole explants showed better results compared to leaf explants, which was 54.4% compared to 32.8% (Fig. 2). The percentage of callus formation was different. It indicates that the ability of petiole and leaf explant of geranium was different.

After it was induced in MS medium by the addition of several concentrations of Benzyl Adenine, the percentage of geranium callus formation increased significantly. The addition of PGR BA on MS medium combined with NAA was able to produce a better callus formation percentage of petiole and leaf explant compared to the percentage of callus formation on MS media with NAA addition (control). The percentage of callus formation on the medium containing 0.1 mg.L^{-1} NAA was 20% of petiole explant and 8% of leaf explant, whereas the percentage of callus formation on the medium with the addition of 0.5-2 mg.L^{-1} BA combined with 0.1 mg.L^{-1} NAA was 52-80% of petiole explant and 24-52% of leaf explant.

The effect of different concentrations of PGR BA could affect the percentage callus formation of geranium. The percentage of callus formation on the medium with the addition of 1 mg.L^{-1} BA with a combination of 0.1 mg.L^{-1} NAA indicated the best results and increased significantly (± 4 times higher than the control) equaled 80% of petiole explants and 52% of leaf explants (Fig. 3).

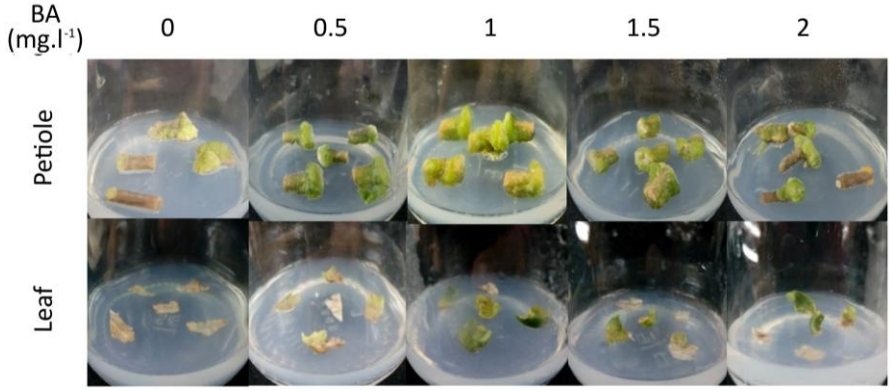


Figure 1. Response of callus growth from petiole and leaf explants on the medium with the addition of plant growth regulator 0.1 mg.L^{-1} NAA combined with several concentrations of BA

ISSN. 2087-2852
E-ISSN. 2338-1655

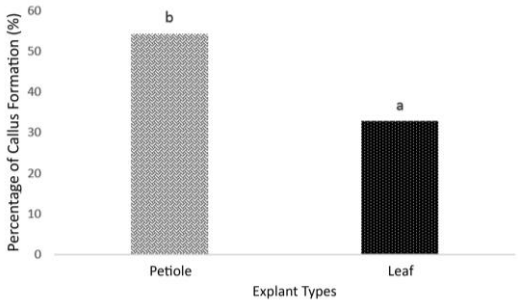


Figure 2. Effect of explant types on percentage of formation of geranium callus at week 4 of culture. **Note:** The same lowercase notation shows no significant effect (DMRT P <0.05)

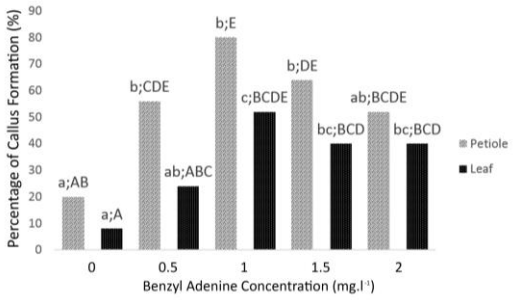


Figure 3. Effect of explant type and various concentrations of PGR BA combined with 0.1 mg.L^{-1} NAA on the percentage of geranium callus formation. **Note:** The same lowercase notation indicate no significant effect between different PGR BA concentrations, while the same uppercase notation indicates no significant effect between different types of explants (DMRT P <0.05)

The different responses of the formation and growth of different callus of petiole and leaf explants could be influenced by the physiological conditions of each explant. Different explants indicated different cleavage responses [14]. Several factors, such as plant genotypes, explant sources, mediums, and plant growth regulators on the medium, influenced formation and growth of callus [15]. Younger tissue and actively splitting explants provided faster and more responsive callus formation [16,17].

Besides being influenced by the type of explants, the formation and growth of callus were also influenced by the balance of plant growth regulators used. Benzyl adenine was a stable and effective synthetic cytokinin to accelerate callus development and growth [18]. The combination of suitable plant growth regulators was a major factor in the success of callus culture [10]. The difference in frequency of callus formation could be influenced by the balance of concentration of plant growth regulators used [17].

The addition of exogenous plant growth regulators into the medium increased the concentration of endogenous hormones in the cell. It can trigger the process of growth and tissue development [19]. The right combination of plant growth regulators (auxin and cytokinin) will stimulate cell division [8]. Plant growth regulators exclusively regulate the division and growth of plant cells. It served to trigger multiplication callus cells that were important for the production of secondary metabolites [20].

CONCLUSION

Geranium callus formation could be induced from petiole and leaf explants. In the 2nd week, the geranium callus was initiated. The percentage of callus formation of petiole explants (54.4%) was better than that of leaf explants (32.8%). The addition of PGR BA combined with NAA could increase the percentage of callus formation from petiole and leaf explants. Benzyl Adenine, with a concentration of 1 mg.L^{-1}, was able to form callus with the highest percentage of explants petiole (80%) and leaf explants (52%).

REFERENCES

[1] Saxena, G., C.V. Praveen, R. Laiqur, B. Suchitra, R.S. Shukla, K. Sushil. 2008. Selection of leaf blight-resistant *Pelargonium graveolens* plants regenerated from callus resistant to a culture filtrate of *Alternaria alternata*. *Crop Prot*. 27. 558-565.

[2] Benslima, A., B.A. Manel, B. Mohamed, T. Al-Ibrahim, B. Tahia, A. Noureddine, G. Radhouane. 2013. Antioxidant properties of *Pelargonium graveolens* L'Her essential oil on the reproductive damage induced by deltamethrin in mice as compared to alpha-tocopherol. *Lipid Health Dis.* 12(30). 1-9.

[3] Rabuma, T. 2015. In vitro propagation of geranium (*Pelargonium graveolens* L.) from nodal culture. *J. Agr. Sci. Tech*. 4(2). 23-34.

[4] Palupi, A.D., Sholichatun, D.M. Soerya. 2004. The effect of 2,4-D and BA on essensial oil production from neem (*Pogostemon cablin* Benth.) callus culture. *BioSMART*. 6(2). 99-103.

[5] Purnamaningsih, R., M. Ashrina. 2011. The effect of BAP and NAA on callus induction and artemisinin content of *Artemisia annua* L. *Berita Biologi.* 10(4). 481-489.

[6] Decruse, S.W., A. Gangaprasad, S. Seeni, M.V. Sarajini. 2003. Micropropagation and ecorestoration of *Vanda spathulata*, an

ISSN. 2087-2852
E-ISSN. 2338-1655

exquisite orchid. *Plant Cell Tiss. Org. Cult.* 72. 199-202.

[7] Miyashita, T., O. Takafumi, S. Fukashi, A. Hajime, H. Yoichiro. 2009. Plant regeneration with maintenance of the endosperm ploidy level by endosperm culture in *Lonicera caerulea* var. emphyllocalyx. *Plant Cell Tiss. Org. Cult*. 98(3). 291-301.

[8] Widiastoety, D. 2014. Effect of auxin and cytokinin on the growth of *Mokara* orchid plantlets. *J. Hort*. 24(3). 230-238.

[9] Erisen, S., M. Yorgancilar, E. Atalay, M. Babaoglu, A. Duran. 2010. Callus induction and plant regeneration of the endemic *Astragalus nezaketae* in Turkey. *Electron. J. Biotechn*. 13(6). 1-7.

[10] Juan, L., W. Lihua, L. Jing, W. Junhuy. 2010. Effect of different plant growth regulators on callus induction in *Catalpa bungei. Afr. J. Agric. Res.* 5(19). 2699-2704.

[11] Zia, M., R. Rehman, M.F. Chaudhary. 2007. Hormonal regulation for callogenesis and organogenesis of *Artemisia absinthium* L. *Afr. J. Biotechnol.* 6(16). 1874-1878.

[12] Tarrahi, R., F. Rezanejad. 2013. Callogenesis and production of anthocyanin and chlorophyll in callus cultures of vegetative and floral explants in *Rosa gallica* and *Rosa hybrida* (Rosaceae). *Turk. J. Bot.* 37. 1145-1154.

[13] Rosmaina, Zulfahmi, P. Sutejo, Ulfiatun, Maisupratina. 2015. Callus Induction of *Eurycoma longifolia* Jack by leaf and petiole explant. *Jurnal Agroteknologi*. 6(1). 33-40.

[14] Savita, V., G.S. Virk, A. Nagpal. 2010. Effect of explant type and different plant growth regulators on callus induction and planlet regeneration in *Citrus jambhiri* Lush. *Environ. We. Int. J. Sci. Tech.* 5. 97-106.

[15] Kumari, S., R.K. Pandey, K. Uttam. 2015. In vitro callus induction from two different explants stem and leaf in *Charthamus tinctorius* Linn. *Eur. J. Exp. Biol*. 5(2). 1-4.

[16] Zulkarnain. 2011. Kultur jaringan tanaman. Bumi Aksara. Jakarta.

[17] Durrani, N.S., D. Ahmad, A. Jalal, H. Rajab, M.S. Khan. 2017. The effect of explant sources and growth regulators on callus induction and regeneration in different tomato cultivars. *J. Anim. Plant Sci*. 27(2). 481-489.

[18] Indah, P.N., D. Ermavitalani. 2013. Induksi kalus daun nyamplung (*Calophyllum inophyllum* Linn.) pada beberapa kombinasi konsentrasi *6-Benzylaminopurine* (BAP) dan *2,4-Dichlorophenoxyacetic Acid* (2,4-D). *Jurnal Sains dan Seni Pomits.* 2(1). E-1 – E-24.

[19] Lestari, E.G. 2010. Peranan zat pengatur tumbuh dalam perbanyakan tanaman melalui kultur jaringan. *Jurnal Agrobiogen*. 7(1). 63-68.

[20] Adil, M., X. Ren, D.I. Kang, L.T. Thi, B.R. Jeong. 2018. Effect of explant type and plant growth regulators on callus induction, growth and secondary metabolites production in *Cnidium officinale* Makino. *Mol. Biol. Rep.* 45. 1919-1927.

ISSN. 2087-2852
E-ISSN. 2338-1655

Detection of Reproductive Status in Ongole Crossbred (PO) Cow Based On Vaginal Epithel Morphology and Profile Hormone

Yeni Widyaningrum[1], Aulanni'am Aulanni'am[2], Agung Pramana Warih Marhendra[2*]

[1]Master Program of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia

Abstract

Hormonal fluctuations in livestock will affect vaginal cytology good overview on the condition of estrous until pregnancy. The purpose of this study was to determine the physiological condition of Ongole crossbred (PO) cow during estrous and determine pregnancy by the description of vaginal epithelial cells, progesterone, and estrogen hormone profiles. The materials were used 35 cows with physiological status (estrous, 5th pregnancy period, 16th pregnancy period, 22nd pregnancy period, and 60th pregnancy period). Samples of Vaginal smear were stained with Giemsa, then it was observed using a microscope, with 40 times magnification. The progesterone and estrogen were analyzed by the ELISA method. The parameters measured were the percentage of vaginal epithelial cells, such as (parabasal, intermediate, and superficial) started estrous phase until the time of pregnancy in cows (5, 16, 22, and 60 days), hormone concentration, as well as the presence or absence of leukocytes. The result showed the Ongole crossbred cow estrous phase percentage of superficial cells 56.27%±6.49 higher than 26.23%±7.98 intermediate cells, followed by parabasal cells 17.50%±4.74. While in Ongole crossbred that were 5t^{h} pregnancy period until the 60th predominantly intermediate cell 80.43%±1.31, then the superficial cells 18.09%±1.30 and 1.48%±0.04 parabasal cells. Progesterone concentration was 63.74±1.07 ng.mL^{-1} in estrus cows, and steadily increased 93.71±0.94 ng.mL^{-1} to 149.5±0.71 ng.mL^{-1} in pregnant cows (5-60 days). The concentration of high estrogen levels were 122.38 ± 0.63 ng.mL^{-1} in the estrous phase, then decreased 81.54±0.44 ng.mL^{-1} in the pregnancy phase. In conclusion, the concentration of hormone showed a diagnosis of pregnancy, which done by looking at changes in vaginal epithelial cells at the Ongole crossbred cow, and the cow estrous phase showed greater superficial cells compared by pregnant cows (5-60 days).

Keywords: diagnosis of pregnancy, estrous, hormone, Ongole crossbred of cow, vaginal cytology.

INTRODUCTION

The accuracy of the detection of estrous in Ongole cow is one of the critical success factors for mating success and high pregnancy rates for breeding animals. Detection of estrous in cows can be seen through animal behavior, body temperature, and external genital organs condition [1]. However, each individual of the Ongole crossbred cow can show the response changes in sexual behavior. So it is not enough to determine the appropriate time for the marriage. Mating success in livestock will be characterized by the occurrence of pregnancy. During this time, many Ongole cow repeat breeding, and also considered to be pregnant but are not parturition. Therefore, the diagnosis of pregnancy needs to know as early as possible.

Generally, pregnancy diagnosis can be determined by Rectal palpation examination and ultrasonography (USG). However, in its application of this method has two weaknesses, new rectal palpation can be performed on the 45th day of gestation and ultrasonography on the 30th. Further, the application field assistant jobs require reproduction (ATR) experienced to ensure pregnancy [2].

Based on that condition by observing changes in vaginal epithelial cells as detection estrous cycle until the time of pregnancy. There have been many studies done using vaginal smears during the estrous cycle in sheep [3], monkeys, deer, and bears to know the estrous cycle [4]. However, little is known about this research in cows, especially Ongole cow.

According to Johnston *et al.* [5] on the estrous phase of vaginal epithelial cells are superficial and cornification cells. The luteal phase vaginal epithelial cells transformed into cells parabasal. It happens because of the hormonal control during the estrous phase (follicular) Gonadotropin-releasing hormone (GnRH), which is a peptide hormone secreted by the hypothalamus tropic. GnRH stimulates the release of FSH and LH from the anterior pituitary gland. FSH stimulates the growth of follicles to produce estrogen. The luteal phase formed the corpus luteum, which produces the hormone progesterone, so that the

* Correspondence address:
Agung Pramana Warih Marhendra
E-mail : agung_pramana@ub.ac.id
Address : Dept. Biology, University of Brawijaya, Veteran Malang, Malang 65145.

endometrium is ready to accept implantation and ended in pregnancy [6]. Seeing the problems and potential in an increase in cattle reproduction Ongole cow, then we research to determine the physiological conditions that occur in estrous phase/mating properly and pregnancy diagnosis through cytological changes in vaginal epithelial cells, as well as progesterone and estrogen hormone fluctuations.

MATERIAL AND METHOD

This research conducted by farmer in the village of the district Nguling Grati, Pasuruan, and cage experiments at Beef Cattle Research, Animal Diseases Diagnostic Laboratory (ADD Lab). It started in February to April 2019.

Material Research

This study used 35 Ongole crossbred (PO) cow, which divided into five groups based on the physiological status of livestock, namely as follows. Group I cows that are not pregnant (estrous), group II pregnant cow day 5 after artificial insemination (AI), Group III to 16-day post-AI, Group IV on day 22 post-AI, and group V day 60 post-AI. The use of cows for the research was approved by the ethics committee, Brawijaya University (No. 1140-KEP-UB).

Vaginal smears

The pillowcase vaginal sample collection is done every day for one estrous cycle, that is by making a smear of the vagina. Vaginal smear was taken using sterile cotton (cotton swab) soaked with physiological saline. Subsequently, swabs smeared on glass objects until it forms a thin review and aerated. We reviewed the vaginal epithelium, which is dry, then fixed in methanol for 5 minutes. Furthermore, staining with Giemsa 10% for 45 minutes [7]. Then washed with running water and dried in air. Mixture swab examined under a microscope with a magnification of 40 times for the observation of the percentage of the number of vaginal epithelial cells.

Observations were made based on the number of 300 cells in each preparation were observed. Vaginal epithelial cells observed then calculated according to the group of each estrous cycle phase that has been determined. Criteria for determining the physiological state based on the epithelial cell shape changes (Table 1) [8].

Progesterone/Estrogene Serum Measurement

ELISA was used to measure the concentration of progesterone and estrogen serum ($ng.mL^{-1}$). A commercial kit was used (Cusabio Technology LLc; Bioassay Technology Laboratory, Cat.No.E0240Bo). A collection of blood 10 mL from the jugular vein for the examination of progesterone concentrations conducted during the estrous, pregnant cow day 5 after artificial insemination, 16-day post-AI, day 22 post-AI, and day 60 post-AI. Confirmation of pregnancy by rectal palpation 45 days after AI. Serum recovered by centrifugation (15 minutes at 4000 rpm) and stored at -20°C until being assayed [9].

Parameter

The criteria phase of the estrous cycle and pregnancy is determined based on the percentage of the epithelial cell morphology picture. The diestrous phase is not formed on the cells of the superficial, proestrous phase cells are found intermediates and the percentage of superficial cells increased. Then the estrous phase of the vaginal epithelial cells formed many superficial/cornification.

Parameters measured were the percentage of the vaginal epithelium (superficial, intermediate, parabasal), the presence or absence of leukocytes cells, and the concentration of progesterone and estrogen hormone.

Data Analysis

Data were analyzed descriptively.

Table 1. Criteria for determining the physiological state based overview of the epithelial cell lines

Epithelial cells	Epithelial cells form	Phase
Superficial cells	Polygonal-shaped cells / flat without a core, Cytoplasm great discount, and the edges of the cell such as folding	Estrous (Follicular)
Intermediate cells	Large cell with a small core	Pregnant (Luteal)
parabasal cells	Cells are round, small and large core	Pregnant (Luteal)

RESULT AND DISCUSSION

Vaginal Epithelial Cells

Based on observations of vaginal epithelial cells in the Ongole cow indicates that there are parabasal, superficial, and intermediates cells [10]. The observation of the vaginal smear shown in Figure 1. Percentage of epithelial cells estrous cows successively showed superficial cells 56.27%, 26.23% intermediate cells, and parabasal 17.50% (Table 2).

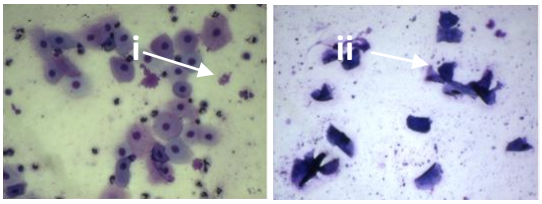


Figure 1. Cytology of vaginal swab with Giemsa staining at PO cow estrous phase (Leukocyte (i), and superficial cells (ii)

Based on the reproductive status of the estrous percentage of superficial cells are more dominant and have found their neutrophil cells. The increase in superficial cells along with higher estrogen concentration in the estrous phase is 122.38±0.63 ng.mL^{-1}, with the lowest concentration of progesterone 63.74±1.07 ng.mL^{-1} (Fig. 3, Table 1). It is consistent with previous studies Junaidi [11] that the phase of the estrous increase superficial cell and concentration of estrogen in the blood is high. The results of the previous study showed that estrogen hormone concentrations increased at the peak of estrus, which was 223.13±9.50 ng.mL^{-1}, then decreased in the metestrus phase 10.05±98.03 and diestrus 67.37±8.75 ng.mL^{-1} [9].

A study reported that in cow, an increase in the superficial cell of the early estrous little, medium, and then very much during peak estrous [12]. Similar results also reported by Schuttle [13] stated that the superficial cells in the vaginal swab as a specific marker by the concentration of estrogen. Superficial cells out of the basement membrane and evolved from other cells, then the cells will mature as happened cornification [14].

According to Najamudin *et al.* [4], when once estrus discovered, many cells form flat and do not have a nucleus is due to lysis. Core lysis of the epithelial cells happened due to keratinization to separate cells. Changes in vaginal epithelial cells are affected by the concentration of the 17-β estradiol hormone [15]. It is because of the 17-β estradiol receptors found in the vagina [14].

Table 2. Analysis of vaginal epithelial cytology ongole cattle in estrous phase and pregnant

Reproductive Status	Superficial cells (%)	Cells Intermediates (%)	Parabasal cells (%)
Estrous phase	56.27± 6.49	26.23 ± 7.98	17.50 ± 4.73
Gestation initial phase (day-5)	41.78± 0.90	56.60 ± 0.89	1.62 ± 0.31
Gestation initial phase (day-16)	42.62 ± 0.82	56.66 ± 0.82	0.72 ± 0.69
Gestation initial phase (day-22)	22.11 ± 4.95	77.19 ± 5.25	0.70 ± 0.68
Gestation initial phase (day-60)	18.09 ± 1.30	80.43 ± 1.31	1.48 ± 0.04

During the estrous phase, the hormone estrogene plays an important role, which will be active in the uterus wall. It causes hypersecretion in epithelial cells of the uterus and vagina. So the superficial cells are followed by the vaginal mucosa [9]. Increasing concentrations of estrogen in the estrus phase may be related to the high percentage of superficial cells. Estrogen is a steroid hormone that is responsible for the growth and regulation of the female reproductive system and secondary sex characteristics.

The hormone estrogen is secreted by granulosa cells from de Graff follicles that are stimulated by FSH [6]. The optimal estrogen hormone causes increased activity of the uterus wall. It is resulting in uterine and vaginal epithelial cell hypersecretion and keratinization. Therefore superficial cells were found on vaginal smear [4]. Based on Suraatmadja's research [16], the hormone estrogen also stimulated the formation of keratohyalin grains, and then it functioned as the center of intracellular filament disintegration (keratinization). Reviews of vaginal cows taken on estrus showed an increased number of cornified cells [17]. Meanwhile, in Bligon goats the percentage of superficial cells was 32.25% with estradiol concentration 247.77 pg dL [18].

The number of superficial cells in the estrous phase serves to protect the vaginal mucosa during copulation [4]. It is also characterized by a large amount of mucous secretion in the vagina. In estrus sheep, vaginal epithelial features also show the dominance of superficial cells [19]. Whereas, upon entering pregnancy, the

ISSN. 2087-2852
E-ISSN. 2338-1655

dominant epithelial cells are intermediate, and no leukocytes are found (Fig. 2).

The results of vaginal examination in pregnant Ongole crossbred cow on day 5, 16, 22, and 60 obtained images of intermediate respectively (56.60±0.89; 56.66±0.82; 77.19± 5.25; 80.43±1.31), superficial (41.78±0.90; 42.62 ±0.82; 22.11±4.95; 18.09±1.30), and parabasal cells (1.62±0.31; 0.72±0.69; 0.70±0.68; 1.48± 0.04). According to Hussain and Khan [20] in young, mid, and late pregnant cows, the dominant percentage of vaginal epithelial cells are intermediate cells 81.63%, 85.9%, and 88.23%. But with increasing gestational age, days 250 to 260, there was a significant increase in the number of superficial cells. It is due to the activity of the hormone estrogen, due to the high concentration of the hormone progesterone [21].

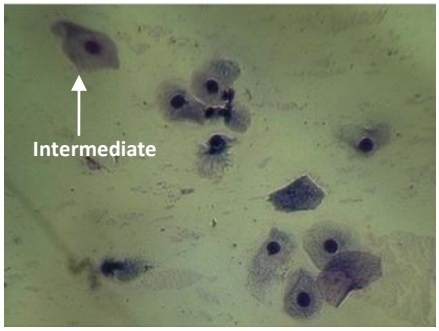


Figure 2. Intermediate cell in cows with young gestation

Progesterone and Estrogen Hormone

Based on the analysis of progesterone, the lowest concentration in the estrous phase is 63.74±1.07 ng.mL^{-1}, and the concentration reaches a peak in the luteal phase. In pregnant cows, the level of the hormone progesterone on the 5th day was 93.71±0.94 ng.mL^{-1}, which seemed to continue to increase slowly until the gestational age was around 60 days 149.05±0.71 ng.mL^{-1} (Fig. 3).

The hormone progesterone plays a role in pregnancy. The hormone progesterone produced by the corpus luteum will inhibit FSH so that no estrous back. The role of the hormone progesterone is maintaining the condition of the uterus to support a pregnancy, implantation, and fetal development [22]. The intermediate cell was found in the histology of vaginal wall epithelial cells. It indicates that the cows are in a pregnancy condition.

This result is consistent with the results of research of Ola *et al.* [23] a vaginal smear during pregnancy in elephant is dominated intermediate cells. Hussain [24] also reported that no leukocytes in the vaginal epithelial smear of pregnant cows. That, as well as the increasing domination of intermediate cells, can also be used as a method to diagnose pregnancy.

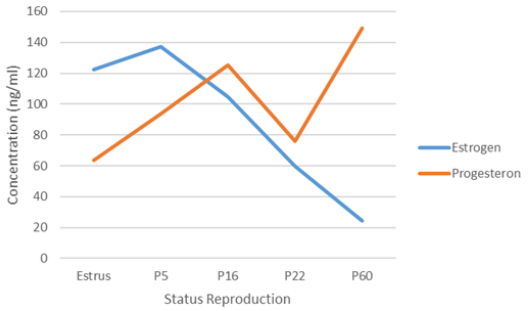


Figure 3. Progesterone and Estrogen Concentration in cows serum

CONCLUSION

Along with the hormonal analysis of estrogen and progesterone, pregnancy diagnosis can be done by looking at changes in vaginal epithelial cells of the Ongole crossbred cow. The epithelial cells dominated by intermediate cells, and in the estrous phase, Ongole crossbred cows show greater superficial cells compared to non-pregnant Ongole crossbred cow.

Acknowledgement

Thank you to the technical staff of the Indonesian Beef Cattle Research Institute (Dyah Tuwi Ramsiati, and M. Chanafi) and Mr. Nawer in conducting this research.

REFERENCES

[1] Piccione, G., G. Caola, R. Refmetti. 2003. Daily and estrus rithmicity of body temperature in domestic cattle. BMC Physiology.

[2] Lucy, M., C. Davies, T. Ott. 2007. Regulation of interferon-stimulated genes in peripheral blood leukocytes in pregnant and bred, nonpregnant dairy cows. *J. Dairy Sci*. 90. 274-280.

[3] Bearden, H.J., J.W. Fuquay, S.T. Willard. 2004. Applied animal reproduction, 6th Ed. Pearson Prentice Hall. USA.

[4] Najamudin, Rusdin, Sriyanto, Amrozi, A. Srihadi, L.Y. Tuty. 2010. Penentuan siklus estrus pada kancil (*Tragulus javanicus*) berdasarkan perubahan sitologi vagina. *Jurnal Veteriner.* 11(2). 81-86.

[5] Johnston, S.D., M.R. Kustritz, P. Olson. 2001. Canine and feline theriogenology. WB Saunders comp. Philadelphia. 32-40.

[6] Julie, E.H, I. Megan. 2017. Physiology, ovulation. NCBI bookshelf. A service of the

ISSN. 2087-2852
E-ISSN. 2338-1655

National Library of Medicine, National Institutes of Health.

[7] Ahmadi, M.R., S. Naziti, H.R. Ghaisari, M. Damchy. 2006. Evaluaion of the cytology of uterus, vagina, and clitoris as predictors of uterine condition in the mare. *Comp. Clin. Pathol.* 14. 186-190.

[8] Nalley, W.M.M., R. Handarini, M. Rizal, R.I. Arifiantini, T.L. Yusuf, B. Purwantara. 2011. Determinaion of the estrous cycle based on vaginal sitology changed and hormon profile in Timor Hind. *Jurnal Veteriner.* 12. 98-106.

[9] Siregar, T.N., J. Melia, Rohaya, C,N. Thasmi, D. Masyitha, S. Wahyuni, J. Rosa, Nurhafni, B. Panjaitan, Herrialfian. 2016. Determining proportion of exfoliative vaginal cell during various stages of estrus cycle using vaginal cytology techniques in Aceh Cattle. Vet. Med. Int. Article ID 3976125. DOI: 10.1155/2016/3976125.

[10] Mayor, P., H. Galvez, D.A. Guimaraes, G.F. Lopez, M. Lopez. 2005. Serum estradiol-17â, vaginal cytology and vulval appearance as predictors of estrus cyclicity in the female collared peccary (*Tayassu tajacu*) from the eastern Amazon region. *Anim. Reprod. Sci.* 97. 165-174.

[11] Junaidi, A. 2005. Reproduksi dan obstetri pada anjing. Gadjah Mada University Press. Yogyakarta.

[12] Subramanian, A., S.R. Pattabiraman. 1988. Exfoliative vaginal cytology in bovines. *Indian J. Anim. Sci.* 58. 209-211.

[13] Schuttle, A.P. 2010. Technique and cytology morphology. *J. Small. Anim. Pract.* 18:301-306.

[14] Bologna, J.W. 2001. Genitourinary problems associated with menopause. *Anim. Reprod. Sci.* 90. 51-55.

[15] Marcondes, F.K., F.J. Bianchi, A.P. Tanno. 2002. Determination of the estrous cyclephase of rats: some helpful considerations. *J. Brazilian Archiv. Biol. Technol.* 4(A). 600-614.

[16] Suraatmadja, T.O. 1982. Cornification process in the vaginal epithelium of the laboratory rat (*Rattus norvegicus*) under the influence of oestradiol valerate: an electron microscope study. Master Thesis. Bogor Agricultural University. Bogor.

[17] Salisbury, G.W., N.L. Vandenmark, J.R. Lodge. 1978. The reproductive system of the cow. In: physiology of reproduction and artificial insemination of catle, 2nd Ed. WH. Freeman and Company. San Fransisco. 35-51.

[18] Widiyono, P.P., P. Putro, Sarmin, Astuti, C.M. Airin. 2011. Kadar estradiol dan progesteron serum, tampilan vulva dan sitologi apus vagina kambing bligon selama siklus birahi. *Jurnal Veteriner.* 2(4). 263-268.

[19] Solis, G., J.I. Aguilera, R.M. Rincon, R. Banuelos, C.F. Arechiga. 2008. Characterizing cytology (ECV) in ewes from 60 d of age through parturition. *J. Anim. Sci.* 82. Suppl. 1.

[20] Hussain, M.P., A.C. Khan. 1979. Exfoliative cytology and biopsy of vagina in pregnant cows. *Kerala J. Vet. Sci.* 10(2). 230-233.

[21] Sharma, M., N. Sharma. 2016. Vaginal cytology: an historical perspective on its diagnostic use. *Adv. Anim. Vet. Sci.* 4(6). 283-288.

[22] Abdullah, M., T.K. Mohanty, M. Bhakat, A. Kumaresan, T.K. Atbandha, A.R. Madkar, A.K. Mohanty. 2017. Metabolic indicators for early pregnancy in zebu and crossbred Dairy cows reared in a subtropical climate. *Turk. J. Vet. Anim. Sci.* 41. 407-41. DOI: 10.3906/vet-1602-10.

[23] Ola, S.I., W.A. Sanni, G. Egbunike. 2005. Exfoliative vaginal cytology during oestrus cycle of west affican dwarf goats. *Reprod. Nutr. Dev.* 46(1). 87-95.

[24] Hussain, A.M. 1979. Bovine uterin defence mechanism: A review. *J. Vet. Med. B.* 36. 641-651. DOI: 10.1111/j.14390450.1989. tb00657.

ISSN. 2087-2852
E-ISSN. 2338-1655

The Artificial Bee Colony (ABC) Algorithm for Estimating Parameter of Epidemic Influenza Model

Ririn Nirmalasari*, Agus Suryanto, Syaiful Anam

Department of Mathematics, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia

Abstract

The Artificial Bee Colony (ABC) is one of the stochastic algorithms that can be applied to solve many real-world optimization problems. In this paper, The ABC algorithm was used to estimate the parameter of the epidemic influenza model. This model consists of a differential system represented by variations of Susceptible (S), Exposed (E), Recovered (R), and Infected (I). The ABC processes explore the minimum value of the mean square error function in the current iteration to estimate the unknown parameters of the model. Estimating parameters were made using participation data containing influenza disease in Australia, 2017. The best parameter chosen from the ABC process matched the dynamical behavior of the influenza epidemic field data used. Graphical analysis was used to validate the model. The result shows that the ABC algorithm is efficient for estimating the parameter of the epidemic influenza model.

Keywords: ABC, Epidemic, Estimate, Influenza, Parameter.

INTRODUCTION

Parameter estimation is the process of using sample data to estimate the parameters of the optimization problem. Several parameter estimation methods are available. In this study, the chosen method is the stochastic method because of its advantages that can find the global minima solution. The determination of parameter values will affect the estimation results. If the parameter values are incorrect, the estimation results will not be accepted. It means that only the best parameter values can use so that the model can represent the field data.

The Artificial Bee Colony (ABC) algorithm is one of the most popular and widely used stochastic methods to find the solution of optimization problems. This algorithm is based on the behavior of bees to find and exploit food resources efficiently [1]. The advantages of this algorithm are simple, reliable, and flexible. The performance of the ABC algorithm is better than other stochastic algorithms such as Particle Swarm Optimization, Genetic Algorithm, and Differential Evolution on constraints or non-constraints [2].

The parameter estimation is widely studied and developed by researchers, in general, using test functions such as the Rastrigin, Ackley, Sphere, and Rosenbrock. Some researchers began to develop and apply the ABC algorithm to estimate parameters of the model in the system of the differential equation, both linear and nonlinear. Roeva [3] used the ABC algorithm to identify the parameters of the cultivated *E. coli* bacteria model.

The aim of this study is the implementation of the ABC algorithm used to estimate the unknown parameters of the influenza epidemic model. The used mathematical model is based on the research of Samsuzzoha [4]. This model is a system of nonlinear differential equations represented by variations of Susceptible (S), Exposed (E), Infected (I), and Recovered (R) populations.The solution of this model was estimated by influenza laboratory surveillance data obtained from WHO regional databases. The chosen laboratory data is based on the well-defined standard condition.

MATERIAL AND METHOD

Epidemic Influenza Model

There are four groups of population in this model: the susceptible population who can contract influenza, the exposed population who affected influenza but not to be infectious, the infected population who has infected and able to transmit the influenza virus, and the recovered population who can be re-infected by the disease if the immunity has been lost. It is assumed that the infected population only affects the susceptible population. However, among these populations, there is an exposed population that lies in a latent period or incubation period and has a low infection rate. Furthermore, the infected population that has received treatment and recovered naturally changes into a recovered population. At one time, this population could be

*Correspondence address:
Ririn Nirmalasari
E-mail : nirmala_ririn@student.ub.ac.id
Address : University of Brawijaya, Veteran Malang, 65145

ISSN. 2087-2852
E-ISSN. 2338-1655

re-infected by influenza when the immunity disappeared. The epidemic influenza model was expressed in the form of the following system of nonlinear differential equations [4],

$$\begin{aligned} \frac{dS}{dt} &= -\beta\frac{IS}{N} - \mu S + rN + \delta R \\ \frac{dE}{dt} &= \beta\frac{IS}{N} - (\mu + \sigma + k)E \\ \frac{dI}{dt} &= \sigma E - (\mu + \alpha + \gamma)I \\ \frac{dR}{dt} &= kE + \gamma I - \mu R - \delta R \end{aligned} \quad (1)$$

and

$$N = S + E + I + R \quad (2)$$

where the parameter description and variable model can be seen in Table 1.

Table 1. Description of all parameters and variables

Parameter	Description
β	Transmission coefficient
σ^{-1}	Incubation period
γ^{-1}	Mean duration of recovery infected population
δ^{-1}	Loss of immunity period
μ	Natural mortality rate
r	Birth rate
k	Recovery rate
α	The disease induced mortality rate
Variable	**Description**
S	Susceptible population
E	Exposed population
I	Infected population
R	Recovered population
N	Total population

The ABC Algorithm Implemented in Epidemic Influenza Model

In the ABC algorithm, a bee colony composes of three groups. Those are employed bee, onlooker bee, and scout bee. The number of bees in a colony is expressed as population size ($Popsize$). Half of $Popsize$ is employed bee, and the other half is onlooker bee. There is one employed bee in each food source. It is said that the number of food sources where are around the nest ($NFood$) is equal to the number of employed bees[5].

The first step in the ABC algorithm is initialization. In this step, there are several processes: inputting parameters, setting boundary conditions, searching initial position of the food source, creating an initial population, calculating objective and fitness function, determining the initial best particle, and setting the $limit$. A food source position represents a potential parameter (x_i) as a solution to the influenza model. The initial food source position was searched randomly by maximum ($xmax$) and minimum ($xmin$) range of parameters using equation (3). The amount of nectar in the food source determines the quality of the solution. It can be calculated by the objective function (MSE) and the fitness value, both expressed in equation (5) and (6).

In the employed bee phase, each bee modified the position of the food source randomly based on neighboring principles. It means that the chosen food source must be different from the previous food source. A new food source was generated from the previous food sources. Furthermore, these food sources are selected using a greedy selection process expressed in equation (9)–(10) to get the best food source ($pbest_i$).

In the onlooker bee phase, a bee was placed in each food source using a roulette wheel selection process, which given in equation (11). This process was based on proportional probabilities to the amount of nectar. Food sources that have the most nectar will be chosen more often by onlooker bee. Furthermore, the onlooker bee updates the food source using the same process in the employed bee phase.

Each bee colony has a scout bee that has no guidance during food search. This bee searched randomly in all directions and found a variety of food sources that have not been visited by an employed bee. It was selected by using a parameter called a limit. If a solution represents a food source that does not produce improvements in some iterations ($trial_i$), the food source discarded by the employed bee, and this bee will be a scout bee. The food sources obtained in each iteration compared with solutions in the previous iteration. If the new food source has a good quality compared to the previous food source, the new food source will be taken as the best solution ($pbest_i$). In the ABC algorithm, onlooker bee and employed bee run the exploitation process. This process applies a piece of existing knowledge to seek the best solution. On the other hand, a scout bee controls the exploration process that concerned for the entire search space of an optimal solution.

The final step is the criteria for stopping iteration. If the process has been reached maximum iteration, the process must be stopped, and the algorithm produces the best solution from all iterations ($gbest$). Based on the above explanation, the process of the ABC

ISSN. 2087-2852
E-ISSN. 2338-1655

algorithm implemented in the epidemic influenza model is given below [6].

Step 1: Initialization

Input parameters t (period of disease), $Popsize$ (number of population), $NFood$ (number of food source), $maxiter$ (maximum iteration), and D (dimensionality of search space);

Set the boundary condition of the parameters ($xmax$ and $xmin$);

for i = 1,2, ..., $NFood$ do

Position for the initial food source using equation (3);

$$x_i(0) = xmax + rand.(xmax - xmin) \quad (3)$$

where $rand$ is a random number [0,1];

Create an initial population using the SEIRS model (equation (1)-(5)) and the Runge-Kutta 4th order method given as below;

$$k_1 = h.f(t_i, x_i)$$
$$k_2 = h.f(t_i + \frac{h}{2}, x_i + \frac{k_1}{2})$$
$$k_3 = h.f(t_i + \frac{h}{2}, x_i + \frac{k_2}{2})$$
$$k_4 = h.f(t_i + h, x_i + k_3)$$
$$x_{i+1} = x_i + \frac{1}{6}.(k_1 + 2k_2 + 2k_3 + k_4) \quad (4)$$

where $f(t_i, x_i)$ is model of influenza, h is interval of time, and x_{i+1} is the solution of model;

Calculate objective functions using MSE;

$$f_i = \frac{1}{t}\sum_{i=1}^{t}(data_i - x_i)^2 \quad (5)$$

Calculate fitness values using equation (6);

$$fit_i = \begin{cases} \frac{1}{1+f_i}, & jikaf_i > 0 \\ 1 + abs(f_i), & jikaf_i < 0 \end{cases} \quad (6)$$

end for;

Determine the initial $pbest$ and $gbest$;

Set iteration = 0;

Set $trial$ = 0;

Set $limit = NFood\ x\ D$; (7)

Step 2: The employed bee phase

for i = 1,2, ..., $NFood$ do

Update new food sources using equation (8);

$$X_i(iteration + 1) = x_i(iteration) + \psi.(x_i(iteration) - x_k(iteration)) \quad (8)$$

where k = 1,2, ..., $NFood$ and ψ is random number [-1,1];

Determine the position of food sources to stay within the range;

Generate new populations using the SEIRS model (equation (1)) and the Runge-Kutta 4th order method (equation (4));

Calculate objective functions using MSE;

Determine fitness values using equation (6);

Select the best food source between x_i and X_i using the Greedy process below,

If $fit_{i+1} > fit_i$, then

$$x_i(iteration + 1) = X_i(iteration + 1) \quad (9)$$
$$trial_i = 0$$

else

$$trial_i = trial_i + 1 \quad (10)$$

end if

end for

Step 3: Calculate P_i Opportunities using equation (11)

$$P_i = \frac{fit_i}{\sum_{j=1}^{NFood} fit_j} \quad (11)$$

Step 4: The onlooker bee phase

for i = 1,2, ..., $NFood$ do

if rand (0,1) <P_i, then

Do Employed bee phase

end if

end for

Step 5: The scout bee phase

if $max(trial_i) < limit$, then

Replace x_i with the new value using equation (3);

Generate x_i population using the SEIRS model (equation (1)) and the Runge-Kutta 4th order method;

Calculate objective functions using MSE;

Determine fitness values using equation (6)

end if

If x_i is better than $pbest_i$, then

Set x_i to $pbest_i$

end if

Determine the particle with the smallest MSE to be the best particle;

$$iteration = iteration + 1 \quad (12)$$

Step 6: If the iteration reached the maximum iteration, the process was stopped. Instead, the process should be repeated in step 2.

ISSN. 2087-2852
E-ISSN. 2338-1655

RESULT AND DISCUSSION

Setting of experiment

The ABC algorithm was coded in Matlab R2013a, and the experiment was implemented 10 times with a different number of populations. It is applied to estimate the parameter of the epidemic influenza model based on infected influenza data in Australia, 2017. This data was presented in Table 3.

The maximum iteration was set to 1000 iterations. The initial condition of parameters and populations showed in Table 2.

Table 2. The initial condition of parameters and populations

Parameter	Value	Source
$Popsize$	**25, 50, 75, 100**	**Assumption**
t	52 (week)	[7]
Dimension (D)	4	Assumption
N	24585 (thousand)	[8]
r	0.0248 (per 100 population per week)	[8]
μ	0.01269 (per 100 population per week)	[8]
σ	[1.75, 7] (per week)	[9]
γ	[1, 1.4] (per week)	[9]
α	0.000098 (per 100 population per week)	[10]
δ	0.0192 (per week)	[4]
β	[4.06e-6,50] (per 100 population per week)	Assumption
k	[4.06e-6,50] (per 100 population per week)	Assumption
S_0	24584.372 (thousand)	Assumption
E_0	0.557 (thousand)	Assumption
I_0	0.036 (thousand)	[8]
R_0	0.035 (thousand)	Assumption

The model of influenza has eight parameters. There are four fixed parameters, namely r, μ, α, and δ, based on Table 2. On the other hand, four unknown parameters, which are σ, γ, β, and k, will be estimated using the ABC algorithm. The initial condition of populations was determined based on Australia Influenza Laboratory Surveillance Information in the first week and several other assumptions.

Model validation

Data in Table 3 was simulated using the ABC algorithm, and the estimation results can be seen in Figure 1 and Figure 2. Figure 1 shows the ability of the ABC algorithm to find global minima solutions. It has been seen that during the searching process until the maximum iteration, the ABC algorithm search for the smallest MSE value of each iteration. The ABC algorithm produces a different solution for each experiment. It due to the selection of population or prospective solution was selected randomly. The convergence of the ABC algorithm was faster when the population sizes were large. It can be seen in Table 4, which smallest fitness value lays in the fifth experiment in population size of 100.

The best parameter value that corresponds to the best fitness values of each $Popsize$ was entered in equation (1), and the results can be seen in Figure 2. Most of the simulation results close to field data values, although some data are difficult to reach by the ABC algorithm. The standard deviation value of each experiment with different $Popsize$ is always less than the mean of the best solution and decreases with increasing $Popsize$. It means that the estimation data was not too diverse, even though the standard deviation value is high due to the high value of the infected population. However, overall the estimated parameters of the ABC algorithm can represent well the model of the spread of influenza disease, especially in Australia.

Finally, the best parameters from all experiments are $\beta = 2.23$, $\sigma = 5{,}63$, $\gamma = 1.32$, and $k = 3{,}12$ (see in Table 4). β parameter means that 2.23% of population will be affected by influenza in a week. Affected people are in an average incubation period for a day. During this period, 3.12% of the population heals from influenza, while people who don't recover become infected populations. This population heals from influenza after those take medicine and medical treatment for five days.

ISSN. 2087-2852
E-ISSN. 2338-1655

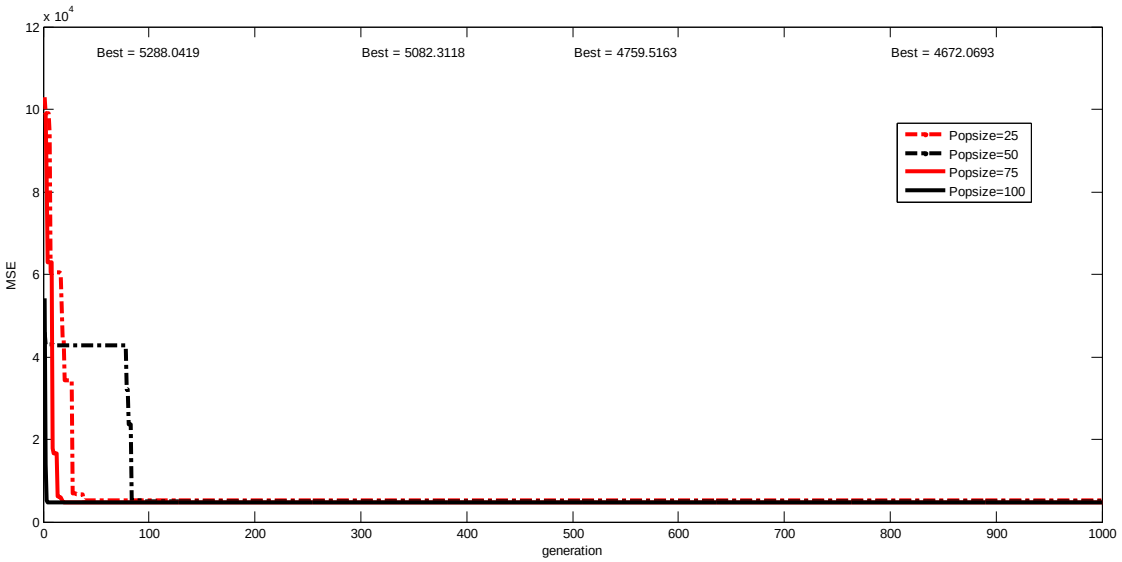


Figure 1. Convergence curves of ABC algorithm

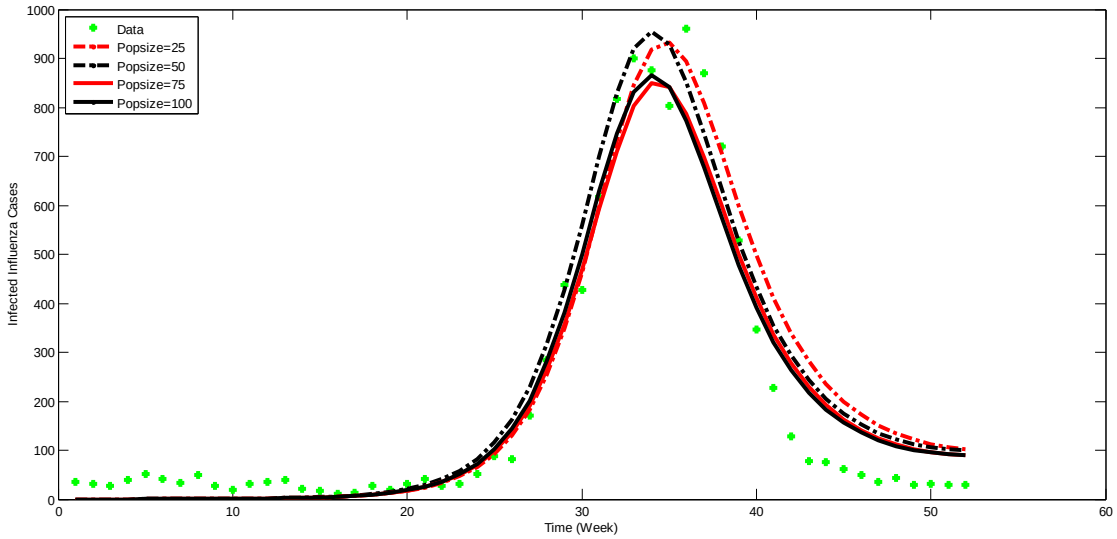


Figure 2. Comparison of field data and estimation results basedon parameter values that correspond to the bestfitness values for each $Popsize$ in Table 4

CONCLUSION

The result shows that the ABC algorithm is efficient for estimating the unknown parameter of the epidemic influenza model. It is due to most of the simulation results are close to field data values. It means that the simulation result represents well the real condition of the epidemic influenza. The population size is influential in obtaining more improved for estimated parameters. Our future study will be focused on the implementation of an improved ABC algorithm or another stochastic method on the dynamical model.

REFERENCES

[1] Karaboga, D. 2005. An idea based on honey bee swarm for numerical optimization. Technical Report-Tr06. Turki.

[2] Karaboga, D., B. Akay. 2009. A comparative study of Artificial Bee Colony algorithm. *Appl. Math. Comput.* 214(1). 108-132. Doi: 10.1016/j.amc.2009.03.090.

[3] Roeva, O. 2018. Application of artificial bee colony algorithm for model parameter identification. Innovative Computing: Optimization and Its Applications, Studies in Computational Intelligence. 741. DOI: 10.1007/978-3-319-66984-7_17.

[4] Samsuzzoha, M., M. Singh, D. Lucy. 2011. Numerical study of diffusive epidemic model of influenza with variable transmission coefficient. *Appl. Math. Model.* 35. 5507-5523.

[5] Suyanto. 2017. Particle swarm optimization. Swarm Intelligence, Modern Computation

for Optimization Big Data Mining. Chapter 4. 51-91.

[6] Li, Z., W. Wang, Y. Yan, Z. Li. 2015. PS-ABC: a hybrid algorithm based on particle swarm and Artificial Bee Colony for high-dimensional optimization problems. *Expert Syst. Appl.* 42(22). 8881-8895. DOI: 10.1016/j.eswa.2015.07.043.

[7] WHO. Influenza: FluNet. 2019. Available at: https//www.who.int/influenza/gisrs_laboratory/flunet/en/.

[8] United Nations, Department of Economic and Social Affairs, Population Division. 2019. World Population Prospects 2019, Volume I: Comprehensive Tables (ST/ESA/SER.A/426). United Nations

[9] WHO. Influenza (seasonal). 2019. Available at: https://www.who.int/en/news-room/fact-sheets/detail/influenza-(seasonal).

[10] Australian Bureau of Statistics. 2017. Australia's leading causes of death, 2017. Available at: https://www.abs.gov.au/ausstats/abs@.nsf/Lookup/by%20Subject/3303.0~2017~Main%20Features~Australia's%20leading%20causes%20of%20death,%202017~2.

ISSN. 2087-2852
E-ISSN. 2338-1655

Table 3. Influenza Laboratory Surveillance Information in Australia, 2017

Year	Start date	End date	Total number of influenza positive viruses	Start date	End date	Total number of influenza positive viruses
2017	2017-01-02	2017-01-08	36	2017-07-03	2017-07-09	170
2017	2017-01-09	2017-01-15	32	2017-07-10	2017-07-16	284
2017	2017-01-16	2017-01-22	28	2017-07-17	2017-07-23	437
2017	2017-01-23	2017-01-29	39	2017-07-24	2017-07-30	428
2017	2017-01-30	2017-02-05	51	2017-07-31	2017-08-06	619
2017	2017-02-06	2017-02-12	41	2017-08-07	2017-08-13	817
2017	2017-02-13	2017-02-19	33	2017-08-14	2017-08-20	901
2017	2017-02-20	2017-02-26	50	2017-08-21	2017-08-27	876
2017	2017-02-27	2017-03-05	27	2017-08-28	2017-09-03	804
2017	2017-03-06	2017-03-12	20	2017-09-04	2017-09-10	960
2017	2017-03-13	2017-03-19	32	2017-09-11	2017-09-17	869
2017	2017-03-20	2017-03-26	36	2017-09-18	2017-09-24	721
2017	2017-03-27	2017-04-02	40	2017-09-25	2017-10-01	529
2017	2017-04-03	2017-04-09	21	2017-10-02	2017-10-08	347
2017	2017-04-10	2017-04-16	18	2017-10-09	2017-10-15	228
2017	2017-04-17	2017-04-23	11	2017-10-16	2017-10-22	128
2017	2017-04-24	2017-04-30	14	2017-10-23	2017-10-29	78
2017	2017-05-01	2017-05-07	27	2017-10-30	2017-11-05	76
2017	2017-05-08	2017-05-14	19	2017-11-06	2017-11-12	62
2017	2017-05-15	2017-05-21	32	2017-11-13	2017-11-19	49
2017	2017-05-22	2017-05-28	41	2017-11-20	2017-11-26	35
2017	2017-05-29	2017-06-04	27	2017-11-27	2017-12-03	43
2017	2017-06-05	2017-06-11	31	2017-12-04	2017-12-10	30
2017	2017-06-12	2017-06-18	51	2017-12-11	2017-12-17	32
2017	2017-06-19	2017-06-25	88	2017-12-18	2017-12-24	30
2017	2017-06-26	2017-07-02	82	2017-12-25	2017-12-31	29

Data source: FluNet (www.who.int/flunet), Global Influenza Surveillance and Response System (GISRS) [7].

Table 4. The Performance of ABC Algorithm

Popsize	Test	Fitness Value	Best	Worst	Mean	Standard Deviation	Parameter Value			
							β	σ	γ	k
25	I	6152.66	5288.04	10915.2	7835.29	2073.42	2.69	3.19	1	4.19
	II	6288.43					2.59	1.75	1.29	1.29
	III	9377.17					2.91	7	1	10.88
	IV	10317.4					2.85	7	1.06	9.92
	V	9935.48					3.00	5.20	1	8.43
	VI	5288.04					2.05	7	1.23	3.72
	VII	6317,16					2.49	7	1.12	6.99
	VIII	7337.14					2.02	6.86	1.13	4.18
	IX	6424.23					2.65	3.43	1.13	3.63
	X	10915.2					2.65	5.06	1.4	3.82
50	I	5082.31	5082.31	10930.9	7535.53	2070.4	2.07	5.57	1.27	2.69
	II	8991.52					2.19	3.95	1.00	3.47
	III	6166.98					2.22	5.48	1.07	4.57
	IV	9040.99					2.85	7	1.03	10.16
	V	6166.98					2.22	5.48	1.07	4.57
	VI	10930.9					2.10	3.50	1.05	2.53
	VII	5082.31					2.07	5.57	1.27	2.69
	VIII	8991.52					2.19	3.95	1.00	3.47
	IX	8991.52					2.19	3.95	1.00	3.47
	X	5910.21					2.53	6.17	1	7.45
75	I	4759.52	4759.52	10140.6	5727.19	1602.13	2.39	3.24	1.32	2.04
	II	5784.37					2.42	6.89	1	7.71
	III	5263.1					2.35	5.97	1.21	4.55
	IV	4759.52					2.39	3.24	1.32	2.04
	V	5366,74					2.27	6.87	1.09	5.86
	VI	5263.1					2.35	5.97	1.21	4.55
	VII	5912.36					2,48	7	1	8.19
	VIII	4759.52					2.39	3.24	1,32	2.04
	IX	10140.6					2.97	7	1	11.26
	X	5263.1					2,35	5.97	1.21	4.55
100	I	6042.76	4672.07	7085.33	5913.48	853.29	2.52	6.81	1.06	7.61
	II	5517.69					2.39	6.35	1.05	6.45
	III	5176.03					2.25	3.56	1,20	2.34
	IV	7085.33					2,12	4.55	1.12	3.07
	V	4672.07					2.23	5.63	1.32	3.12
	VI	5300.55					2.31	4.79	1.11	4.06
	VII	6940.62					2.67	5.87	1.04	7.40
	VIII	5300.55					2.31	4.79	1.11	4.06
	IX	6940.62					2.67	5.87	1.04	7.40
	X	6158.6					2.71	3.51	1	4.66

ISSN. 2087-2852
E-ISSN. 2338-1655

Combination *Moringa oleifera* Extract and Ifalmin as Potential Formulation of Preventing Inflammation in Diabetic Mice Model

Ruri Vivian Nilamsari[1], Wahyu Isnia Adharini[1], Noviana Dwi Lestari[1], Hideo Tsuboi[2], Muhaimin Rifa'i[1*]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang,
[2]Department of Immunology, Nagoya University Graduate School of Medicine, Nagoya, Japan

Abstract

In Indonesia, the prevalence of diabetes mellitus hits 6.2%, making Indonesia one of the top ten diabetes mellitus countries. Efforts to prevent and treat people with diabetes in Indonesia are required to minimize that as well. One is through treatment with local herbal products such as *Moringa oleifera* (MO) and Toman fish extract (*Channa micropeltes*), called Ifalmin. The aim of this research is to investigate the potential role of a combination of Extract *Moringa oleifera* and Ifalmin to reduce inflammation in diabetes conditions. Diabetic mice were done by Streptozotocin (STZ) induction with a single-dose 145 $mg.kg^{-1}$.Then, diabetic mice were given an oral treatment of combination MO extract and Ifalmin for 14 days. In this experiment combinations of MO extract and Ifalmin are divide into 3 dose, There are: dose 1 (800 $mg.kg^{-1}$: 800 $mg.kg^{-1}$), dose 2 (650 $mg.kg^{-1}$: 650 $mg.kg^{-1}$), and dose 3 (800 $mg.kg^{-1}$: 650 $mg.kg^{-1}$). Immune cells originate from the spleen are stained by immunofluorescence antibodies and analyzed by flow cytometry with BD Cellquest ProTM software. The results showed an increase of expression pro-inflammatory cytokines IL-1β and IL-6 in diabetic mice compared to normal control. Only dose 1 and dose 2 has shown the capability to reduce the expression of IL-1β in diabetic mice. But, the combination of MO and Ifalmin has an antagonist effect on the expression of IL-6. The inhibitory mechanism can be assumed by the action of antioxidant compounds (Flavonoids and Alkaloids) in MO and Albumin compound in Ifalmin. Those combination act as exogenous antioxidant that help endogenous inside the body. A combination of MO extract and Ifalmin with a certain dosage was able to decrease proinflammatory cytokines IL-1β on the cells involved in innate immunity.

Keywords: Diabetes Mellitus, Ifalmin, Inflammation,Innate Immunity,*Moringa oleifera*.

INTRODUCTION

Diabetes mellitus has been a major problem in Indonesia since the 1980s, with the number of patients exceeding 10 million. Diabetes mellitus is a metabolite disorder that has spread around the world. In Indonesia, the prevalence rate of diabetics hits 6.2%, making Indonesia one of the top ten countries in the world with a relatively high number of diabetics[1]. High glucose condition induced a rise in the number of lymphocytes, macrophage, and cytokine associated with inflammation such as IL-6, IL-1β, and TNF-α, which could adversely affect the body's physiological condition [2,3].

Diabetes also triggered imbalances in homeostasis of immune cells, Th 1 and Th 17 are upregulated, and Th 2 and T regulators are downregulated[4]. Th cell is a mediator to activation of B cell so that they will proliferate and differentiate into plasma cells capable of producing IgG. In addition, activation of B cells will produce proinflammatory cytokines such as IL-6 [5]. Prevention and treatment efforts are needed to reduce people with diabetes in Indonesia. One of them is by treatment using local herbal such as *Moringaoleifera* (MO) and extract derived from Toman fish (*Channamicropeltes*) called Ifalmin. MO contains phytochemical compounds such as polyphenols, phenolic acids, flavonoids, alkaloids, glucosinolates, isothiocyanates, tannins, and saponins [6]. Flavonoids inside MO, such as Quercetin, Kaempferol, Myricetin has beenreported can decrease the level of inflammation and Reactive Oxygen Species (ROS) [6].

Powder from MO seeds reportedly can reduce levels of IL-6 to suppress inflammation caused by high sugar levels [7]. According to Mbikay [8], MO has the potential to reduce high blood sugar in diabetic rats [9]. Ifalmin is a product derived from crude *Channa micropeltes,* and it is a family of Channidae that found in freshwater waters.Toman fish contain albumin, zinc, omega 6, and omega 3 [9]. The albumin content in toman fish is quite high when compared to the Channidae family of 5.35%. Albumin has the potential to decrease the level of inflammation and wound healing. This research aims to investigate the potential role of a combination of *Moringa oleifera* extract and

* Correspondence Address:
Muhaimin Rifa'i
E-mail : rifa123@ub.ac.id
Address :Dept. Biology, University of Brawijaya, Veteran Malang, 65145

ISSN. 2087-2852
E-ISSN. 2338-1655

Ifalmin to reduce inflammation in diabetes conditions [10].

MATERIAL AND METHOD

Animal

The research design on this research followed the ethical clearance granted by the Universitas Brawijaya Research Ethics Committee, Indonesia No: 1180-KEP-UB. A total of 30 male Balb/c mice (*Mus musculus*) aged eight weeks were obtained from pathogen-free breeder Singosari, Malang. Mice were divided into six treatment groups there are healthy group, diabetic mice group, metformin group, and diabetic mice given a combination of MO and Ifalmin extracts with different dosage: Dose 1 (800 $mg.kg^{-1}$: 800 $mg.kg^{-1}$), Dose 2 (615 $mg.kg^{-1}$: 615 $mg.kg^{-1}$), and Dose 3 (800 $mg.kg^{-1}$: 615 $mg.kg^{-1}$). All mice are kept in a pathogen-free condition in the Animal Room, Animal Physiology Laboratory, Biology Department, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia.

Induction of Diabetes Mellitus

Diabetic mice induction uses 145 $mg.kg^{-1}$Streptozotocin (STZ) (Bioworld, USA). This dose is a modification from Brosius [11]. STZ was given once with intraperitoneal injection. Diabetes mice were determined when fasting glucose levels ≥126 $mg.dL^{-1}$ and ≥200$mg.dL^{-1}$ in without fasting.

Moringa oleifera and Ifalmin Preparation

Simplisia of *Moringa oleifera* (MO) leaves (Meteria Medika, Batu, East Java, Indonesia) was extracted by a modified method from Khan *et al.* [12]. MO boiled in water at 80°C, then filtered using filter paper (Whatman No.1) freeze-dried and stored at -20°C. Ifalmin originated from Crude toman fish (*Channa micropeltes*) (PT Ismut Fitomedika Makassar, South Sulawesi, Indonesia). Two ingredients are combined based on a predetermined dosage (Dose 1 (800 $mg.kg^{-1}$: 800 $mg.kg^{-1}$), Dose 2 (615 $mg.kg^{-1}$: 615 $mg.kg^{-1}$), and Dose 3 (800 $mg.kg^{-1}$: 615 $mg.kg^{-1}$). Then, both materials are dissolved in Hydrobath before given to the mice diabetes mellitus group as a treatment every day for 14 days.

Spleen Cell Isolation, Immunostaining, and Flow Cytometric Analysis

After 14 days, the mice were dislocated following the last injection for spleen organ isolation. Then washed the isolated spleen using PBS and Homogenized in PBS. The homogenate was centrifuged (2500 rpm, 10°C, 5 minutes), and for immunostaining and flow cytometry analysis, the pellet obtained was resuspended with 1 mL PBS. A total of 50 µL resuspended splenocytes with 50 µL extracellular antibodies (anti-CD11b) (BioLegend,USA) was added, then incubated at 4°C for 20 minutes. The sample was added after incubation with 50 µL permeabilization buffer (Cytofix) (BioLegend,USA), and incubated at 4°C for 20 minutes.

Sample washed using 400 µL wash buffer permeabilization (Washperm) (BioLegend,USA) and centrifuged (2500 rpm, 10°C, 5 minutes). Pellet added with 50 µL intracellular antibody (BioLegend,USA), and incubated at 4°C for 20 minutes. The sample was added with 400 µl PBS and transferred to a cuvette for flow cytometry analysis (BD FACS Calibur,USA).

Data Analysis

The flow cytometry results were analyzed using the BDCell Quest Pro program (BD Biosciences, USA). The data then were analyzed using one-way ANOVA and followed with the Tukey HSD test to determine the statistical difference among treatments (IBM Statistics, USA). Data were considered significant if $p<0.05$.

RESULT AND DISCUSSION

Immunomodulatory Effect on Relative Number of Proinflammatory CytokinesIL-1β

Diabetic mice group had the highest relative number of IL-1β compared to other groups($p>0,05$). A combination extract of MO and Ifalmin could reduce the relative number of IL-1β in all dosage compared to the Diabetes group ($p>0,05$) (Fig. 1).Diabetescondition causes NFκB activation, so pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α could be secreted. IL-1β is a pro-inflammatory cytokine, which plays a significant role in mediating the expression of various cytokines, adipokines, and other pro-inflammatory chemokines [13]. IL-1β can cause damage to pancreatic β cells throughout the condition of diabetes mellitus [14].

The presence of an interleukin-1 receptor antagonist (IL-1Ra), which is one of the cytokines that inhibit the action of IL-1β through MyD88, can inhibit IL-1β signaling activity. It prevents the development of IL-1β, so inflammation that can destroy pancreatic β cells can be suppressed [15]. High glucose activity increased of M1 but decreased of M2 activity[16]. Activation of M1 due to increased IL-6 expression, and IL-10 decreased[17]. In the case of diabetes mellitus, B-cell secreted pro-inflammatory cytokines are increased, and anti-inflammatory cytokines are decreased [18,19].

ISSN. 2087-2852
E-ISSN. 2338-1655

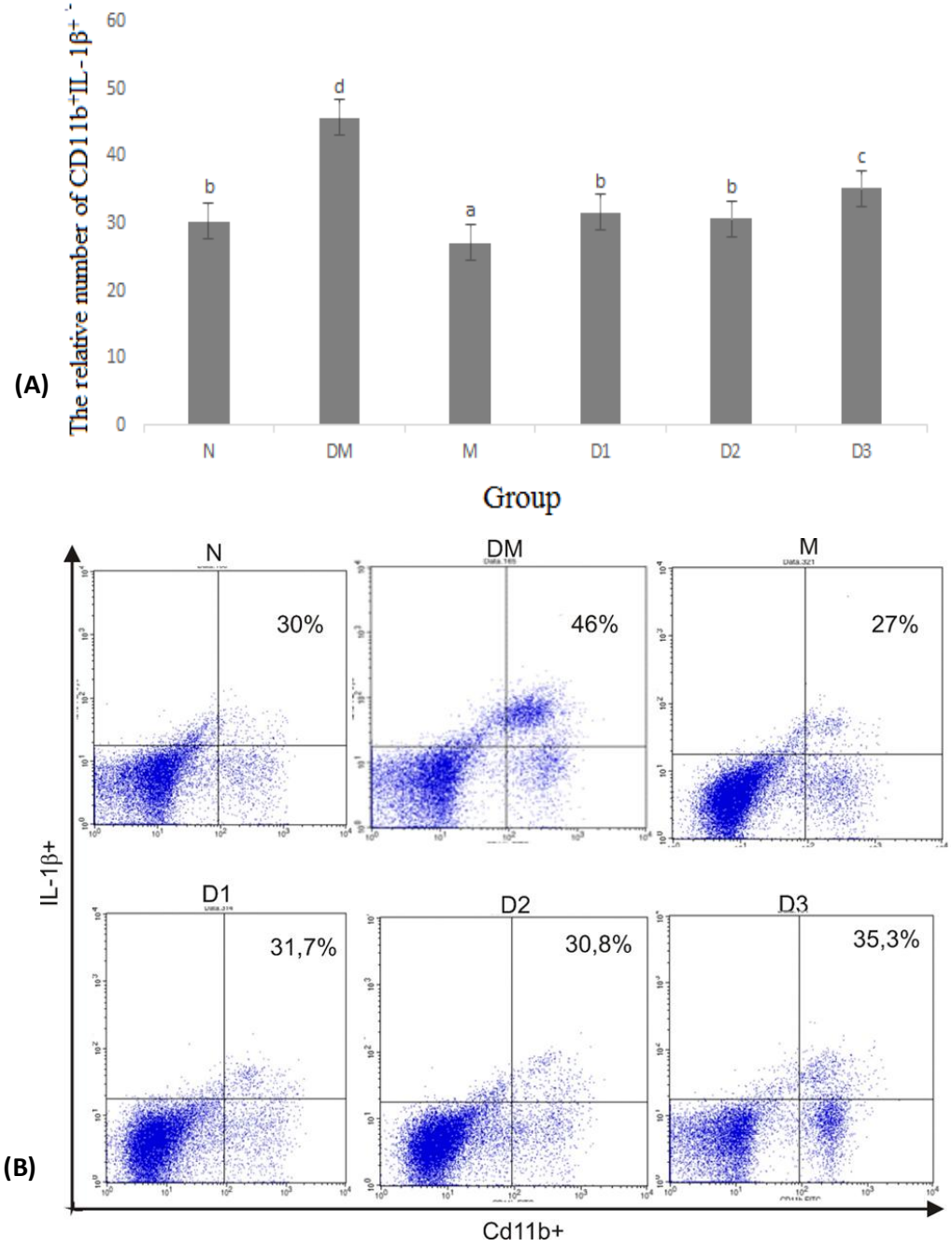


Figure 1. Effect combination extract MO and Ifalmin reduce relative number IL-1β^{+} (CD11b^{+}, IL-1β^{+}) on statisical test (a) Effect combination extract MO and Ifalmin in relative number B Cell and Macrophage on diabetic mice shown in flow cytometry (b). N: Normal, DM: Diabetes Mellitus, M: Metformin, D1: Dosage 1(800 mg.kg^{-1}: 800 mg.kg^{-1}), D2: Dosage 2(615 mg.kg^{-1}: 615 mg.kg^{-1}), D3: Dosage 3(800 mg.kg^{-1}: 615 mg.kg^{-1}). The difference in notations showed significant differences ($p < 0.05$).

Immunomodulatory Effect on Relative Number of Proinflammatory Cytokines IL-6

Meanwhile, combination of extract MO and Ifalmin had no significant effect to reduce relative number of IL-6 ($p > 0,05$) between treatment group (Dose 1 (800 mg.kg^{-1}: 800 mg.kg^{-1}), Dose 2 (615 mg.kg^{-1}: 615 mg.kg^{-1}), and Dose 3 (800 mg.kg^{-1}: 615 mg.kg^{-1}) and diabetes group (Fig. 2). In diabetic rat model, Moringinine showed an increase in glucose tolerance[20]. It is documented that MO seed powder can also reduce IL-6 levels compared to treatment with diabetes control. This decrease in IL-6 profile reflects a decrease in inflammatory activity due to high levels of sugar[7].

ISSN. 2087-2852
E-ISSN. 2338-1655

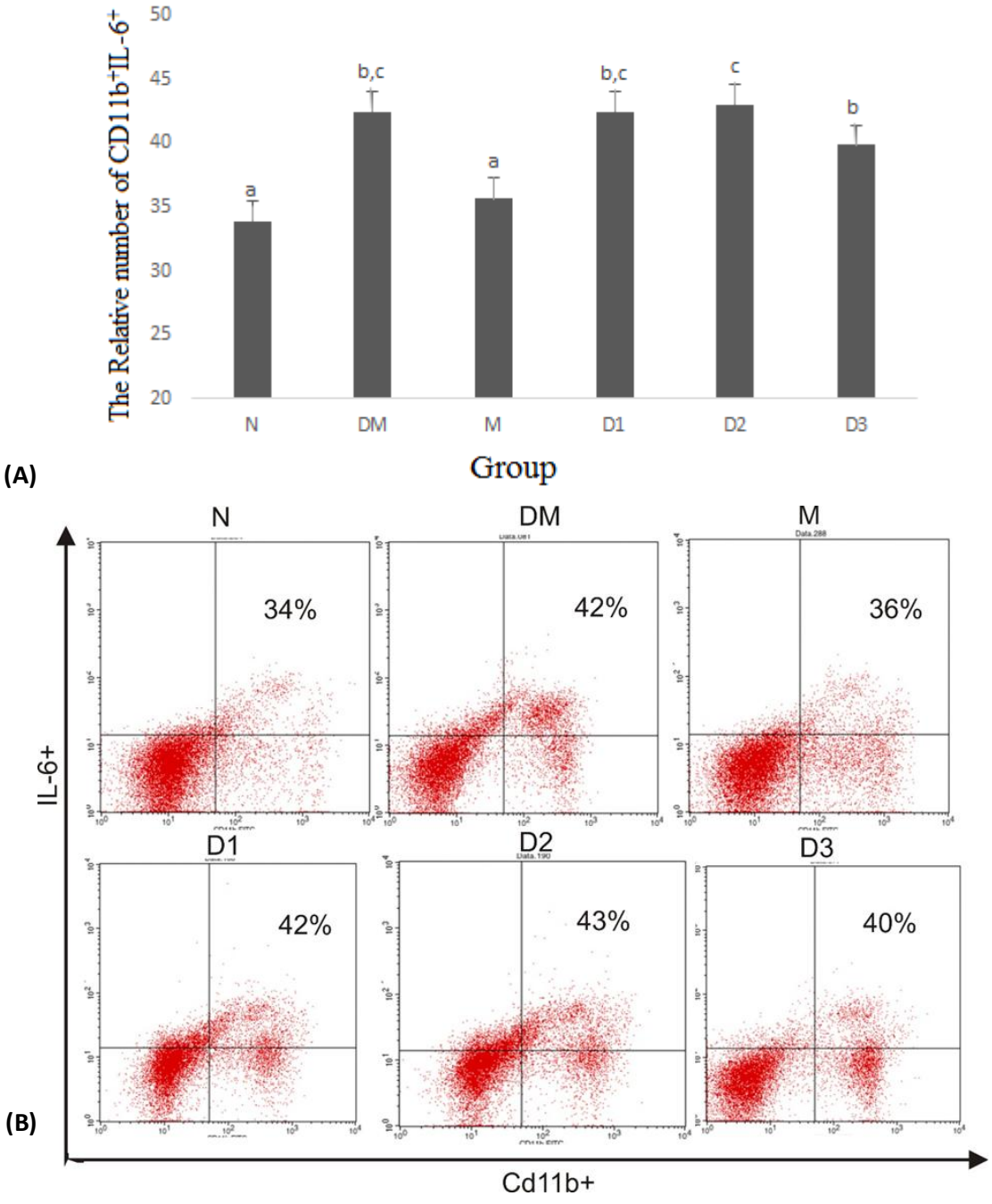


Figure 2. Combination extract MO and Ifalmin reduce relative number gives the opposite effect to IL-6 ($CD11b^+$, $IL\text{-}6^+$) on statisical test (a) Effect combination extract MO and Ifalmin in relative number B Cell and Macrophage on diabetic mice shown in flow cytometry (b). N: Normal, DM: Diabetes Mellitus, M: Metformin, D1: Dosage 1(800 $mg.kg^{-1}$: 800 $mg.kg^{-1}$), D2: Dosage 2(615 $mg.kg^{-1}$: 615 $mg.kg^{-1}$), D3: Dosage 3(800 $mg.kg^{-1}$: 615 $mg.kg^{-1}$). The difference in notations showed significant differences ($p < 0.05$).

Discussion

In this experiment, we showed that a combination of MO extract and Ifalmin (*Channa micropeltes*) can deminish the production of pro-inflammation cytokine such as IL-1β and IL-6. *Moringa oleifera* contains of nutrients that are beneficial to humans, such as calcium, potassium, zinc, magnesium, iron, and copper [21]. Besides minerals, MO also contains several phytochemical compounds, namely polyphenols, phenolic acids, flavonoids, alkaloids, gluco-sinolates, isothiocyanates, tannins, and saponins [22]. There are have been reported to have sufficient amount of these antioxidants and are also used as green leafy vegetables of high total antioxidant capacity (260 $mg.100\ g^{-1}$) [22].

ISSN. 2087-2852
E-ISSN. 2338-1655

MO leaf was thought to be associated with decreased intake of intestinal glucose and slowing down of gastric emptying by fiber in MO leaf. It contained 12% fiber and affected postprandial plasma glucose by three major bioactive phytochemicals, including quercetin, chlorogenic acid, and moringinine [23]. Quercetin, a potent antioxidant, had antidiabetic effects in the insulin resistance model Zucker rat [24]. It has been shown that chlorogenic acid inhibits glucose6-phosphate translocase in rat liver, which has resulted in decreased hepatic gluconeogenesis and glycogenolysis[20]. In human studies, chlorogenic acid during the oral glucose tolerance test showed a decrease in the glycemic response[25].

Meanwhile, Ifalmin has been reported contain high level of Albumin. Albumin from ifalmin can act as antioxidant soit can inhibit formation of ROS [10].Albumin has the potential to reduce the number of macrophages by increasing Th2 cells by secreting IL-10 and TGF-β anti-inflammatory cytokines [26]. Toman fish (*Channa micropeltes*) also contain omega 6, which has derivatives of Arachidonic Acid (AA), which will be converted into lipoxins and have the potential to reduce the risk of inflammation due to diabetes[27].

CONCLUSION

Combination extract *Moringa oleifera* and Ifalmin (Crude *Channa micropeltes*) could decrease production of Pro-inflammatory cytokine IL-1β^{+} in Dosage 1 (800 mg.kg^{-1}: 800 mg.kg^{-1}) and dosage 2 (615 mg.kg^{-1}: 615 mg.kg^{-1}). But there is no significant effect on IL-6^{+}.

Acknowledgment

The authors also thank all colleagues in the Laboratory of Animal Anatomy and Physiology, Biology Department, Brawijaya University. The author also thanks Department of Biology, Faculty Mathematics and Natural Science, Brawijaya University, Indonesia for provided facilities for this research.

REFERENCES

[1] Ligita,T., W. Kristin, F. Karen, H.Nichole, N. Intansari. 2019. How people living with diabetes in Indonesia learn about their disease : A grounded theory study. *Plos One*.3-19.

[2] Fujioka, K. 2007.Pathophysiology of type 2 diabetes and the role of incretin hormones and beta-cell dysfunction. *J. Am. Acad. Phys.*20(12). 3-8.

[3] Foulis, A.K. 2008. Pancreatic pathology in type 1 diabetes in human. *Novartis Found Symp*.292. 2-13.

[4] Zhou, T., H.Zheng, Y.Shou, S.Lin, Y.Zhenxiang, W.Guixia. 2018. Role of adaptive and innate immunity in type 2 diabetes mellitus. *J. Diabet. Res.* Article ID 7457269.1-9.

[5] Szablewski,L. 2014. Role of immune system in type 1 diabetes mellitus pathogenesis. *Int. Immunopharmacol*. 22. 182-191.

[6] Atkinson, M.A., S.E.George, W.M. Aaron. 2014. Type 1 diabetes. *Lancet*.383(9911). 69-82.

[7] Gopalakhrisnan, L., K.Doriya, D.S. Kumar. 2016. *Moringa oleifera*: a review on nutritive importance and its medicinal application. *Food Sci. Human Wellness*.5(2). 49-56.

[8] Mbikay,M. 2012. Therapeutic potential of *Moringa oleifera* leaves in chronic hyperglycemia and dyslipidemia: A review. *Front Pharmacol*.3. 24.DOI: 10.3389/fphar.2012.00024.

[9] Firlianty. 2016. Vacuum drying albumin powder of snakehead (*Channa micropeltes*) potential for wound healing from Central Kalimantan, Indonesia. *Int. J. ChemTech Res.* 9(5). 263-269.

[10] Apriasari,M.L.,A. Yusfa, F. Eka, N.C. Amy. 2018. Antioxidant effect of *Channa micropeltes*in diabetic wound of oral mucosa. *Int. J. Pharmacol.*15 (1). 137-143.

[11] Brosius, F. 2009. High-dose streptozotocin induction protocol (mouse). *Diac. Comp. Consortium*. 12(4). 1-3.

[12] Khan, W., P. Rabe, C. Karisma, P. Shabana, A. Sayeed. 2017. Hypoglycemic potential of aqueous extract of *Moringa oleifera* Leaf and In Vivo GC-MS metabolomics. *Front. Pharmacol.* 8(577). 1-16.

[13] Rehman, K., S.H.K. Muhammad. 2016. Mechanisms of inflammatory responses and development of insulin resistance: how are they interlinked. *J. Biomed. Sci.* 23. Article No. 87.

[14] Zhao, X., Q. Guangfang,W. Yiran, C. Xiao, L. Jiancan, Z.Yaping, H. Qin, W. Qijin. 2016. Elevated B cell activation is associated with type 2 diabetes development in obese subjects. *Cell. Physiol. Biochem.* 1257-1266.

[15] O'Shea,J., M. Gadina, R. M. Siegel. 2019. Cytokines and cytokine receptors. *Immunity Sci.* 127-153.

ISSN. 2087-2852
E-ISSN. 2338-1655

[16] Khairunnida, N.C. Amy, L.A. Maharani. 2018. The effect of giant snakehead(*Channa micropeltes*)extract on the number of macrophage in diabetes melitus wound healing (in vivo study on the back of male wistar rat (*Rattus novergicus*) *Jurnal Kedokteran Gigi.* 3(2). 189-195.

[17] Hirayama,D., I. Tomoya, N. Hirashi. 2017. The phagocytic function of macrophage-enforcing innate immunity and tissue homeostasis. *Int. J. Mol. Sci*. 19. 1-14.

[18] Povlou, S., L. Jamie, I. Rebecca, H. Xu, C. Mei. 2018. Sustained high glucose exposure sensitizes macrophage responses to cytokine stimuli but reduces their phagocytic activity. *BMC Immunol.* 19-24.

[19] Siewko,K., M. Rafal, Z.W. Anna, P.K. Anna, S. Piotr, W.K. Natalia, L. Danuta, M. Robert, G. Maria, K. Adam, S. Malgorzata. 2019. Interleukin-6 and interleukin-15 as possible biomarkers of the risk of autoimmune diabetes development. *Biomed Res. Int.* 1-7.

[20] Rivera, L., R. Moron, M. Sanchez, A. Zarzuelo, M. Galisteo. 2008. Quercetin ameliorates metabolic syndrome and improves theinflammatory status in obese Zucker rats.*Obesity*. 16(9). 2081-2087.

[21] Kasolo, J.N., S.B.Gabriel, O.Lonzy,O. Joseph,W. Jasper, O. Ogwal. 2010. Phytochemicals and uses of *Moringaoleifera* leaves in Ugandan rural communities. *J. Med. Plant Res.*4(9). 753-757.

[22] Georgewill, O.A., U.O.Georgewill, R.N.P.Nwankwoala. 2010. Anti-inflammatory effects of *Moringa oleifera* lam extract in rats. *Asian Pac. J. Trop. Med.*3(2). 133–135.

[23] Jaiswal,D., P. Kumar, R.A. Kumar., S.M.G. Watal. 2009. Effect of *Moringa oleifera* Lam. leaves aqueous extract therapy on hyperglycemic rats. *J. Ethnopharmacol.* 123 (3). 392-396.

[24] Bennett, R.N.,F.A. Mellon, N. Foidl. 2003. Profiling glucosinolates and phenolics in vegetative and reproductive tissues of the multi-purpose trees *Moringa oleifera* L. (Horseradish tree) and *Moringa stenopetala* L. *J. Agric.Food Chem*.51(12). 3546-3553.

[25] Karthikesan, K.,L. Pari, V.P. Menon. 2010. Combined treatment of tetrahydrocurcumin and chlorogenic acid exerts potential antihyperglycemic effect on streptozotocin-nicotinamideinduced diabetic rats.*Gen. Physiol. Biophys.* 29(1). 23-30.

[26] Karthikesan, K.,L. Pari, V.P. Menon. 2010. Antihyperlipidemic effect of chlorogenic acid and tetrahydrocurcumin in rats subjected to diabetogenic agents. *Chemico-Biological Interactions.*188(3). 643-650.

[27] Paolo,C., D.Marco, T.Alessandra, N.Lucia, S.R. Carmen, T.Manuel, B. Mauro. 2013. Clinical indications for the albumin use: Still a controversial issue. *Eur. J. Intern. Med.*24. 721-728.

ISSN. 2087-2852
E-ISSN. 2338-1655

The Role of *Moringa oleifera*- Ifalmin® Formulation in Regulation of $B220^+IgM^+$ and $B220^+IgG^+$ in Diabetic Mice

Wahyu Isnia Adharini, Ruri Vivian Nilamsari, Noviana Dwi Lestari, Nashi Widodo, Muhaimin Rifa'i*

Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia

Abstract

It has been known that the immunoglobulin levels were altered in diabetes mellitus (DM) conditions. This study aimed to evaluate the levels of immunoglobulins in DM mice after the administration of *Moringa oleifera*-Ifalmin® formulation (MI). Streptozotocin, at a dose of 145 $mg.kg^{-1}$, was injected intraperitoneally to experimental mice to obtain diabetic mice. The groups were divided into normal mice, diabetic mice without treatment, diabetic mice with metformin treatment (307.5 $mg.kg^{-1}$ BW), and diabetic mice with MI treatment at dose 1 (M:I= 800 $mg.kg^{-1}$ BW: 800 $mg.kg^{-1}$ BW), dose 2 (M:I= 615 $mg.kg^{-1}$ BW: 615 $mg.kg^{-1}$ BW), and dose 3 (M:I= 800 $mg.kg^{-1}$ BW: 615 $mg.kg^{-1}$ BW). Mice were orally treated by MI for 14 days. Subsequently, the levels of immunoglobulin IgM and IgG were evaluated using flow cytometry analysis. IgM and IgG levels were significantly lower in the DM group than the normal group. These results indicated that DM altered immunoglobulin levels. MI treatment for 14 days significantly increased the number of IgM and IgG at the level equivalent to the normal group and significantly different as compared to the DM group. Based on the results, MI can be used as an immunomodulatory agent in humoral immunity through the precise regulation of IgM and IgG.

Keywords: Diabetes mellitus, Ifalmin®, Immunoglobulin, immunomodulator, *Moringa oleifera*.

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease characterized by high levels of blood glucose (hyperglycemia) [1]. In 2019, the prevalence of DM globally increased to 463 million people, and predictably raised to 700 million in 2045. DM caused 4.2 million deaths in 2019 [2] and was the seventh leading cause of death, according to the World Health Organization (WHO) [3]. In DM, hyperglycemia resulted from a defect in insulin secretion or action [4].

Some studies demonstrated that there was a tight correlation between the immune system and DM pathogenesis [5,6]. The immunoglobulins are essential components of humoral immunity that have an important role in effector function. The immunoglobulin levels are often determined to represent the status of humoral response in clinical practice [7]. The previous study demonstrated that the levels of immunoglobulin changed in the mice model of diabetes [8]. DM is also associated with the alteration of immunoglobulin [7,9].

The use of synthetic hypoglycemic drugs and insulin can cause side effects in DM patients. Furthermore, both drugs and insulin usually target a single pathway of metabolic, which only focuses on hyperglycemia regulation [10]. Therefore, another alternative treatment is needed to minimalize these side effects. Nowadays, herbal medicine becomes a great prospect for alternative treatment in some diseases, including DM.

Moringa oleifera (MO) and Ifalmin® are good sources of nutrition. MO leaves are rich in polyphenols such as flavonoids, phenolic acid, tannins, etc. MO also contains vitamins including vitamin A, E, and C [11] and minerals such as calcium (Ca), zinc (Zn), iron (Fe), potassium (K), manganese (Mn), copper (Cu), and magnesium (Mg) [12]. Ifalmin® is a food supplement from *Channa micropeltes* extract that contains high levels of albumin. Previous studies demonstrated that both MO leaves [13] and albumin [14] have anti-diabetes activities. However, the efficacy of MO and Ifalmin® formulation in the regulation of IgM and IgG in diabetic mice never been studied.

In this study, we used the formulation of MO and Ifalmin® (MI). We assumed that MI, based on their nutritional contents, could be used as an alternative treatment in DM. This formulation was expected to target some mechanisms in DM conditions, including the regulation of altered immunoglobulins. Therefore, this study aimed to evaluate the levels of IgM and IgG in mice models of DM.

MATERIAL AND METHOD

Experimental Design

A total of 30 male mice (*Mus musculus*), strain BALB/c, with the ages between 8-10 weeks were obtained from Singosari Breeder (Malang, Indonesia) and used in this study. The mice

*Correspondence address:
Muhaimin Rifa'i
Email : immunobiology@ub.ac.id
Address : Dept. Biology, University of Brawijaya, Malang 65145, Indonesia.

divided into six groups including the normal group (N) or non-diabetic mice, diabetic mice with metformin administration (DM-M), diabetic mice without MI administration (DM), and diabetic mice with administration of MI dose 1 (DM-D1), dose 2 (DM-D2), and dose 3 (DM-D3). The formulation of used doses were D1 (MO 800 $mg.kg^{-1}$ BW: I 800 $mg.kg^{-1}$ BW), D2 (MO 615 $mg.kg^{-1}$ BW: I 615 $mg.kg^{-1}$ BW) and D3 (MO 800 $mg.kg^{-1}$ BW: I 615 $mg.kg^{-1}$ BW). Each group consisted of five replications. The experiment using the animal model in this study conducted according to ethical clearance approved by The Ethical Committee of Brawijaya University (Reg. No. 1180-KEP-UB).

Induction of Diabetic Mice

A single high dose of Streptozotocin (STZ) used to induce the DM in the mice [15]. The mice fasted for four hours then they were injected by STZ at a dose of 145 $mg.kg^{-1}$ BW mice. The levels of blood glucose were measured from the mice tail using Easy Touch glucometer (Bioptik Technology Inc., Taiwan) after four days of STZ injection. Mice considered diabetic when the levels of blood glucose were higher than 200 $mg.dL^{-1}$ [16].

Oral Administration of MI and Metformin

Five grams of MO leaves powder (Materia Medica Batu, Malang) dissolved in 50 mL of boiled water. Then, it was filtered using Whatman no. 1 paper and subjected to freeze-drying. The extract of MO formulated with Ifalmin®. Ifalmin® obtained from PT. Ismut Fitomedika (Makassar, Indonesia). MI was administrated in diabetic mice (DM-D1, DM-D2, and DM-D3) by oral for 14 days.

Metformin, as a standard drug in this study, was obtained from PT. Hexparm Jaya (Bekasi). Metformin (307.5 $mg.kg^{-1}$ BW mice) dissolved in water. The metformin was administrated in the DM-M group by oral for 14 days. The conversion of human dose to mice equivalent dose in this research is determined based on the Food and Drug Administration (FDA) [17].

Isolation of Spleen

After 14 days of MI treatment, the mice sacrificed by the cervical dislocation technique. Then, the spleen organ was isolated from the mice to obtain the immunoglobulin-expressing lymphocyte B cell. The spleen homogenized in phosphate buffer saline (PBS), and the homogenate moved into microtube, then centrifuged at 2500 rpm, 10°C for 5 minutes. The supernatant removed, and the pellet added by PBS. A total of 50 µL sample moved into a microtube for antibody staining.

Antibody Staining and Flow Cytometry Analysis

The antibodies applied at a concentration of 0.005 $mg.100\ \mu L^{-1}$ based on the company's protocol. The sample containing lymphocyte cells was stained with extracellular antibody before intracellular antibody staining. Staining of extracellular antibody conducted by adding 50 µL of FITC-conjugated rat anti-mouse B220 (Biolegend®, San Diego) into the cells, then incubated for 20 minutes in the icebox (4°C).

Next, the cells were added by 50 µL of cytofix (BD-Biosciences Pharmingen), incubated for 20 minutes in the icebox, and added by wash perm solution (WPS) (BioLegend®, USA), and centrifuged at 2.500 rpm, 10°C for 10 minutes. Then, the supernatant removed from the sample, and each pellet stained with 50 µL of PE/Cy5-conjugated rat anti-mouse IgG (Biolegend, San Diego) and PE/Cy5-conjugated rat anti-mouse IgM (Biolegend, San Diego). The sample was moved into a flow cytometry tube and added with 400 µL of PBS. Flow cytometry tube was put into the flow cytometer (BD FACSCalibur, USA) to run the flow cytometry. The flow cytometry data were analyzed using BD Cellquest Pro™ software (BD Biosciences, San Jose, CA, USA) [18].

Data Analysis

The data were statically analyzed using the SPSS program with one way of variance analysis (ANOVA) and were continued to the Post Hoc test with the p-value <0.05.

RESULT AND DISCUSSION

The results showed that the levels of $B220^{+}IgM^{+}$ (Fig.1) and $B220^{+}IgG^{+}$ (Fig. 2) were significantly lower in the DM group as compared to the normal group ($p<0.05$). The levels of IgM reduced from 14.6% (N) to 9.43% (DM), while the IgG levels decreased from 8.05% (N) to 5.03% (DM). The decreasing levels of IgM and IgG in diabetic mice caused by hyperglycemia conditions, but the mechanism remains unclear [7]. Hyperglycemia in this study results from the cell death mechanism of β-pancreatic after injection of STZ. STZ enters the β-pancreatic cells through glucose transporter 2 (GLUT-2) and induces its toxicity. The toxic effect of STZ is due to the DNA alkylating agent, methyl-nitrosourea (MNU), which causes DNA damage of β-pancreatic cells and eventually induces hyperactivation of poly (ADP-ribose) (PARP) enzyme [19,20].

Hyperactivation of PARP can suppress cell energy reserves (NAD^+) and reduce ATP, thereby inducing necrosis.

Necrosis in β-pancreatic cells will reduce the secretion of insulin, a hormone that plays an important role in glucose metabolism. Thus, insulin lack results in hyperglycemia. Hyperglycemia can prevent the production of IgM in splenocytes [21] and decrease the production of IgM in B lymphocytes cells [8]. Moreover, hyperglycemia can increase hydroxyl radical (OH.) [22,23], one of the reactive oxygen species (ROS) with high reactivity and is known able to damage IgG structure [24] so that it decreases the levels of IgG. The decline of IgM and IgG levels also seen in type 1 and 2 DM [9,25].

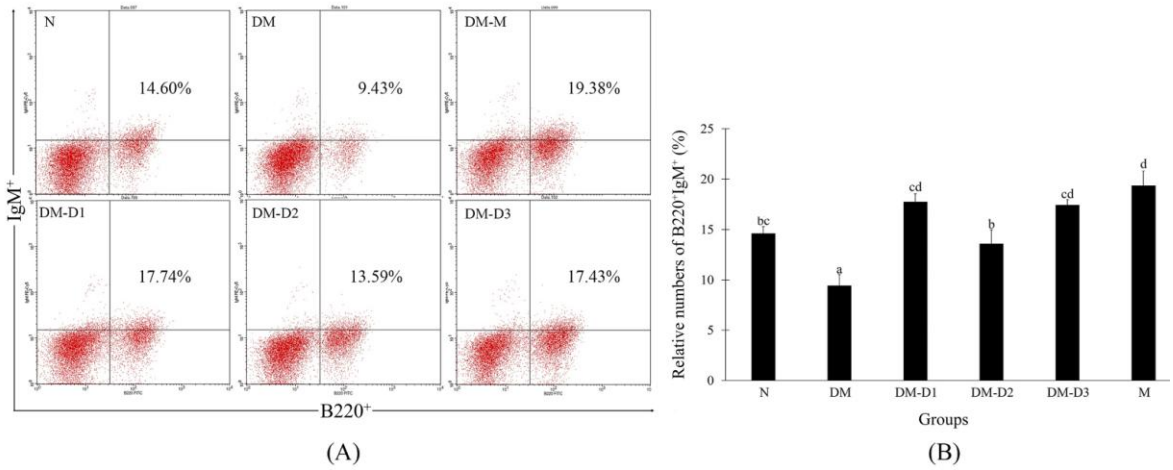


Figure 1. MI administration increased the levels of IgM expressed by $B220^+$-expressing cells in diabetic mice. (A) Spleen cells ($2x10^6$) were obtained from all mice, then subjected to extracellular staining cells with anti-B220 antibody, intracellular staining cells with anti-IgM antibody, and analyzed by flow cytometry. N: non-diabetic mice without STZ induction; DM: diabetic mice with STZ induction without MI treatment; DM-D1: diabetic mice with MI treatment dose 1 (MO 800 $mg.kg^{-1}$ BW: I 800 $mg.kg^{-1}$ BW); DM-D2: diabetic mice with MI treatment dose 2 (MO 615 $mg.kg^{-1}$ BW: I 615 $mg.kg^{-1}$ BW); DM-D3: diabetic mice with MI treatment dose 3 (MO 800 $mg.kg^{-1}$ BW: I 615 $mg.kg^{-1}$ BW); DM-M: diabetic mice with metformin treatment. (B) The bars are a calculation of $B220^+IgM^+$ in splenic cells. The data are mean value ± SD of five mice in each group with a significant value<0.05 (n = 30).

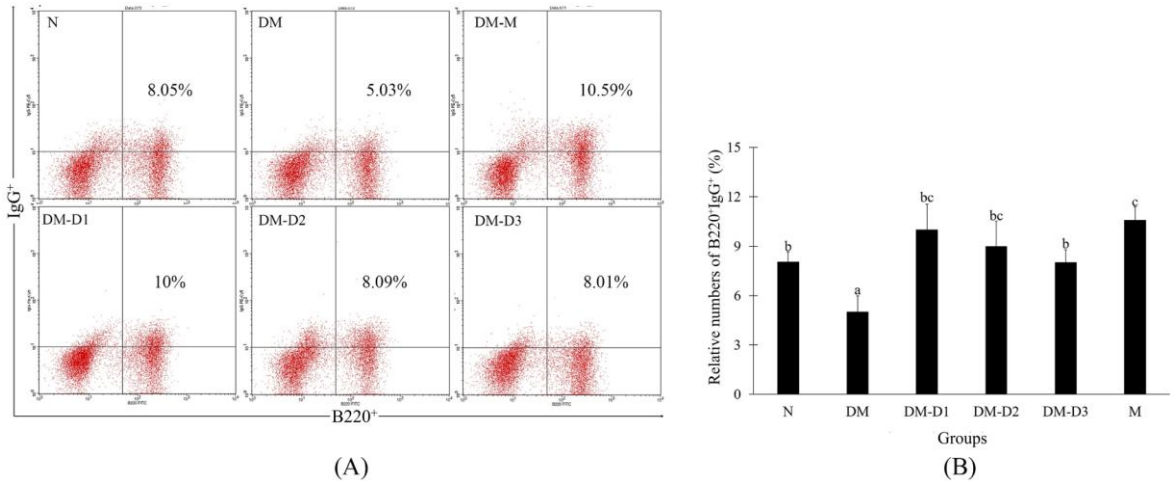


Figure 2. MI administration increased the levels of IgG expressed by $B220^+$-expressing cells in diabetic mice. (A) Spleen cells ($2x10^6$) were obtained from all mice, then subjected to extracellular staining cells with anti-B220 antibody, intracellular staining cells with anti-IgG antibody, and analyzed by flow cytometry. N: non-diabetic mice without STZ induction; DM: diabetic mice with STZ induction without MI treatment; DM-D1: diabetic mice with MI treatment dose 1 (MO 800 $mg.kg^{-1}$ BW: I 800 $mg.kg^{-1}$ BW); DM-D2: diabetic mice with MI treatment dose 2 (MO 615 $mg.kg^{-1}$ BW: I 615 $mg.kg^{-1}$ BW); DM-D3: diabetic mice with MI treatment dose 3 (MO 800 $mg.kg^{-1}$ BW: I 615 $mg.kg^{-1}$ BW); DM-M: diabetic mice with metformin treatment. (B) The bars are a calculation of $B220^+IgG^+$ in splenic cells. The data are mean value ± SD of five mice in each group with a significant value<0.05 (n = 30).

ISSN. 2087-2852
E-ISSN. 2338-1655

IgM and IgG have the immune-regulatory activities in controlling of auto-antibody response during autoimmune diseases, including type 1 DM. These immune-regulatory activities are possibly performed by suppressing the activity of phagocytosis through Fc receptor interaction, thereby preventing the binding of auto-antibody to its cell target [26,27]. The inability of B lymphocyte cells to secrete IgM correlated to impaired B lymphocyte development, which can ultimately lead to the development of autoreactive B cells in mice model of type 1 DM [26]. Furthermore, low levels of IgG and IgM were associated with the prevalence of type 2 DM [7]. Therefore, the decreasing levels of IgM and IgG in this study might indicate the progression of DM.

This study proved that oral administration of MI for 14 days in diabetic mice significantly increased the levels of IgM and IgG. MI administration in diabetic mice increased the levels of IgM from 9.43% to 17.74% (D1), 13.59% (D2), and 17.43% (D3), respectively (Fig. 1). Furthermore, the administration of MI in diabetic mice increased IgG levels from 5.03% to 10% (D1), 8.99% (D2), and 8.01% (D3) (Fig. 2). The administration of MI could increase the levels of IgM and IgG at the levels equal to the normal group and significantly different from the DM group. These results indicated that MI could restore the decreased levels of IgM and IgG to normal levels in diabetic mice.

The abilities of MI to increase the levels of immunoglobulin in diabetic mice represent its immunomodulatory activities in humoral immunity. These immunomodulatory activities of MI might occur through hypoglycemic activity and antioxidant mechanisms as the decreasing levels of IgM and IgG affected by hyperglycemia and stress oxidative. STZ can increase the levels of ROS [28] which also have a destructive effect on β-pancreatic cells. Furthermore, hyperglycemia as well increases the production of ROS and decreases antioxidant enzyme, thereby inducing oxidative stress [29].

MI was assumed to have antioxidant activity due to high flavonoids in MO leaves and abundant albumins in Ifalmin®. Flavonoids exert its antioxidant activity by donating its electron and hydrogen atom to some radicals, including hydroxyl radicals, superoxide, alkoxyl, and peroxyl, thereby stabilizing them [30]. Quercetin in MO leaves can increase the endogenous antioxidant enzymes via the nuclear-related factor 2 (Nrf2) pathway [31]. Another plant with antioxidant properties reported having an immunostimulant effect in IgM and IgG [32]. Furthermore, flavonoid and phenolic acid in MO leaves have hypoglycemic activity through inhibition of amylase enzyme [33]. This enzyme functions to convert the starch into glucose, decrease in intestinal absorption of glucose, and eventually reduce the glucose levels.

Albumin in Ifalmin® is known to activate Nrf2 signaling for gene expression of endogenous antioxidant enzymes, including superoxide dismutase (SOD), heme oxygenase-1 (HO-1), glutathione S-transferase, and glutathione peroxidase [34]. Some studies demonstrated that antioxidant enzymes were decreased in DM conditions [35,36]. Albumin contains high sulfhydryl (-SH) groups that have acted as a radical scavenger to bind ROS, thereby reducing the levels of ROS and preventing stress oxidative. Moreover, anti-hyperglycemic of albumin has been demonstrated by Dwijayanti et al. [14] in mice model of type 2 DM. The efficacy of albumin in decreasing the glucose levels occurred due to its anti-inflammatory activity as the inflammatory mediators in DM cause the dysfunction of β-pancreatic cells.

In this study, we also compared the efficacy of MI with a standard drug, metformin, in the regulation of IgM and IgG. The results showed that the administration of metformin in diabetic mice significantly increased the levels of $B220^{+}IgM^{+}$ (Fig. 1) and $B220^{+}IgG^{+}$ (Fig. 2) as compared to the DM group ($p<0.05$). The increasing levels of IgM and IgG after metformin administration possibly occurs through its anti-hyperglycemia effect. Metformin reduces glucose levels by suppressing the production of hepatic glucose and inhibiting intestinal glucose transport [37]. However, the IgM and IgG levels in the DM-M group were significantly higher than the normal group.

CONCLUSION

Based on the results, we concluded that the formulation of *Moringa oleifera* – Ifalmin® (MI) could be used as an immunomodulator in diabetic mice through precise regulation of IgM and IgG. The oral administration of MI for 14 days in diabetic mice significantly increased the levels of IgM from 9.43% to 17.74% (D1), 13.59% (D2), and 17.43% (D3), respectively. Meanwhile, the administration of MI in diabetic mice increased IgG levels from 5.03% to 10% (D1), 8.99% (D2), and 8.01% (D3).

Acknowledgement

The authors gratefully thank the contributions of the Animal Physiology's staff for assistance in conducted research.

REFERENCES

[1] Baynest, H.W. 2015. Classification, pathophysiology, diagnosis and management of diabetes mellitus. *J. Diabetes Metab.* 6(5). 1-9.

[2] International Diabetes Federation. 2019. IDF Diabetes Atlas, 9th Ed. International Diabetes Federation. Brussels, Belgium.

[3] World Heart Organization (WHO). 2018. Diabetes – Key Facts. Available at: https://www.who.int/news-room/fact-sheets/detail/diabetes.

[4] American Diabetes Association. 2013 American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care.* 37(1). 81-90.

[5] Szablewski, L. 2014. Role of immune system in type 1 diabetes mellitus pathogenesis. *Int. Immunopharmacol.* 22(1). 182-191.

[6] Zhou, T., Z. Hu, S. Yang, L. Sun, Z. Yu, G. Wang. 2018. Role of adaptive and innate immunity in Type 2 diabetes mellitus. *J. Diabetes Res.* 1-9.

[7] Guo, X., G. Meng, F. Liu, Q. Zhang, L. Liu, H. Wua et al. 2016. Serum levels of immunoglobulins in an adult population and their relationship with type 2 diabetes. *Diabetes Res. Clin.Pract.* 115. 76-82.

[8] Jennbacken, K., S. Stahlman, L. Grahnemo, O. Wiklund, L. Fogelstrand. 2013. Glucose impairs B-1 cell function in diabetes. *Clin. Exp. Immunol.* 174. 129-138.

[9] Ali, B.A.M., M.A. Sayed, H.M. Moness, M.M. Mahmoud. 2017. Assessment of serum levels of immunoglobulin A and immunoglobulin G in children newly diagnosed with type 1 diabetes mellitus (T1D). *Curr. Pediatr. Res.* 21(4). 627-631.

[10] Watal, G., P. Dhar, S.K. Srivastava, B. Sharma. 2014. Herbal medicine as an alternative medicine for treating diabetes: The global burden. *Evid-based Complement. Alternat. Med.* 2-4.

[11] Vergara-Jimenez, M., M. Almatrafi, M. Fernandez. 2017. Bioactive components in *Moringa oleifera* leaves protect against chronic disease. *Antioxidants.* 6(4). 1-13.

[12] Lestari, N.D., S. Khalleefah, S. Puspitarini, Widodo, A. Soewondo, M. Rifa'i. 2019. The role of *Moringa oliefera*-Albumin combination as a traditional medicine to control the development of SDF-1 and TER-119+VLA-4+ in diabetes mice models. Proceedings of The 2nd International Conference on Biosciences and Medical Engineering (ICBME 2019): Towards innovative research and cross-disciplinary collaborations.

[13] Adejoh, I.P., O.S. Chiadikaobi, A.O. Barnabas, A.O. Ifeoluwa, H.S. Muhammed. 2016. In Vivo and In Vitro comparative evaluation of the anti-diabetic potentials of the parts of *Moringa oleifera* tree. *Eur. J. Biotechnol. Biosci.* 4(1). 14-22.

[14] Dwijayanti, D.R., M.S. Djati, M. Ibrahim, M. Rifa'i. 2015. The potential of VipAlbumin® to chronic inflammation in type 2 diabetes mellitus Balb/C mice model. *Am. J. Immunol.* 11(2). 56-67.

[15] Diabetic Complications Consortium. 2015. High-Dose Streptozotocin Induction Protocol (Mouse). Animal models of diabetic complications consortium. 4–6.

[16] America Diabetes Association. 2018. Classification and diagnosis of diabetes: standards of medical care in diabetes – 2018. *Diabetes Care.* 4(1). 13-27.

[17] USFDA. 2005. Guidance for industry: estimating the maximum safe starting dose in adult healthy volunteer. US Food and Drug Administration. Rockville, MD.

[18] Rifa'i, M., N. Widodo. 2014. Significance of propolis administration for homeostasis of CD4+CD25+ immunoregulatory T cells controlling hyperglycemia. *Springerplus.* 3. 1-8.

[19] Wu, J., L. J. Yan. 2015. Diabetes, metabolic syndrome and obesity: targets and therapy dovepress Streptozotocin-induced type 1 diabetes in rodents as a model for studying mitochondrial mechanisms of diabetic β cell glucotoxicity. *Diabet. Metab. Synd. Obes. Targets and Therapy.* 8. 181-188.

[20] Zhang, J., L.R. McCabe 2014. Use of Streptozotocin in experimental type 1 diabetic models. In: Gauthier EL, editor. New Developments Medical Research. Streptozotocin uses, mechanism of action and side effects. Nova Science Publishers, Inc. New York. 25-36.

[21] Nikolajczyk, B.S., M. Jagannathan-Bogdan, H. Shin, R. Gyurko. 2011. State of the union between metabolism and the immune system in type 2 diabetes. *Genes Immun.* 12. 239-250.

[22] Frances, D.E., M. T. Ronco, J. A. Monti, P. I. Ingaramo, G.B. Pisani, J.P. Parody et al. 2010. Hyperglycemia induces apoptosis in rat liver through the increase of hydroxyl radical: New insights into the insulin effect. *J. Endocrinol.* 205(2). 187-200.

[23] Ohkuwa, T., Y. Sato, M. Naoi. 1995. Hydroxyl radical formation in diabetic rats induced by streptozotocin. *Life Sci.* 56(21). 1789-1798.

[24] Tripathi, T., Z. Rasheed. 2010. The oxidative by-product, hydroxyl radical, damaged immunoglobulin-G in patients with non-insulin dependent diabetes mellitus. *Bratisl Lek Listy.* 111. 477-484.

[25] Hoddinott, S., J. Dornan, J.C. Bear, N.R. Farid. 1982. Immunoglobulin levels, immunodeficiency and HLA in type 1 (insulin- dependent) diabetes mellitus. *Diabetologia.* 23. 326-329.

[26] Wilson, C.S., P. Chhabra, F. Andrew, Marshall, C. V. Morr, B.T. Stocks, E.M. Hoopes et al. 2018. Healthy donor polyclonal IgMS diminish B-lymphocyte autoreactivity, enhance regulatory T-cell generation, and reverse type 1 diabetes in NOD mice. *Diabetes.* 67(11). 2349-2360.

[27] Matucci, A., E. Maggi, A. Vultaggio. 2015. Mechanisms of action of Ig preparations: Immunomodulatory and anti-inflammatory effects. *Front. Immunol.* 1-5.

[28] Eleazu, C.O., K.C. Eleazu, N. Chukwuma, U.N. Essien. 2013. Review of the mechanism of cell death resulting from streptozotocin challenge in experimental animals, its practical use and potential risk to humans. *J. Diabetes Metab. Disorders.* 12(1). 1-7.

[29] Poljsak, B., D. Suput, I. Milisav. 2013. Achieving the balance between ROS and antioxidants: when to use the synthetic antioxidants. *Oxid. Med. Cell. Longev.* 1-11.

[30] Karak, P. 2019. Biological activities of flavonoids: an overview. *Int. J. Pharm. Sci. Res.* 10(4). 1567-1574.

[31] Ozgen, S., O.K. Kilinc, Z. Selamoğlu. 2016. antioxidant activity of quercetin: a mechanistic review. *Turk. J. Agric. Food Sci. Technol.* 4(12). 1134-1138.

[32] Sadek, K.M. 2012. Antioxidant and immunostimulant effect of Carica papaya Linn. Aqueous extract in acrylamide intoxicated rats. *Acta Inform. Med.* 20(3). 180-185.

[33] Tadera, K., Y. Minami, K. Takamatsu, T. Matsuoka. 2006. Inhibition of α-glucosidase and α-amylase by flavonoids. *J. Nutr. Sci. Vitaminol.* 52(2). 149-153.

[34] Apriasari, M. L., Y. Ainah, E. Febrianty, A.N. Carabelly. 2018. Antioxidant effect of *Channa micropeltes* in diabetic wound of oral mucosa. *Int. J. Pharmacol.* 15(1). 137-143.

[35] Fatima, N., S.M. Faisal, S. Zubair, M. Ajmal, S.S. Siddiqui, S. Moin, M. Owais. 2016. Role of pro-inflammatory cytokines and biochemical markers in the pathogenesis of type 1 diabetes: Correlation with age and glycemic condition in diabetic human subjects. *PLoS ONE.* 11(8). 1-17.

[36] Verma, S., N. Sagar, P. Vats, K.N. Shukla, M. Abbas, M. Banerjee. 2013. Antioxidant enzyme levels as markers for type 2 diabetes mellitus. *Int. J. Bioassays.* 685-690.

[37] Horakova, O., P. Kroupova, K. Bardova, J. Buresova, P. Janovska, J. Kopecky, M. Rossmeis . 2019. Metformin acutely lowers blood glucose levels by inhibition of intestinal glucose transport. *Sci. Rep.* 9(1). 1-11.

Physiological and Genetic Variations of *Amorphophallus variabilis* in Bojonegoro based on Glucomannan Content, Calcium Oxalate and RAPD Markers

Achmad Yogi Pambudi[1*], Nunung Harijati[2], Estri Laras Arumingtyas[3]

[1]Master Program of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia

Abstract

This study had purpose to determine the glucomannan and calcium oxalate content in 7 variants of *A. variabilis* and their genetic relationships based on RAPD markers. *Amorphophallus variabilis* Tuber samples were taken from Karangdowo village, Sumberrejo Sub-district, Bojonegoro District, East Java. Each variant was analyzed for its glucomannan content by the spectrophotometric method using 3,5-DNS reagents and calcium oxalate by the 0.1N KMnO4 titration method. Leaf DNA extraction was carried out using the CTAB method. Relationship analysis used RAPD markers with 5 primers (OPA-11, OPC-04, OPU-06, OPC-07, and OPN-1). The obtained data were analyzed using the Numerical Taxonomy and Multivariate System (NTSYS-pc) version 2.1. The grouping of glucomannan content or oxalium oxalate used hierarchical clustering analysis (SPSS 16.0). This research found that the calcium oxalate content in seven variants of *A. variabilis* ranged from 0.01 to 0.03 g, where the variant with the lowest calcium oxalate is V6 with a value of 0.01 g and the highest is the V7 variant with a value of 0.03 g, while the glucomannan content ranges from 9 - 38%. The highest glucomannan content is V3, while the lowest is V6. Phenograms formed based on RAPD markers showed the formation of two groups of *A. variabilis*. Group one has two subgroups. Subgroup one consists of variants V1 and V4, while subgroup two consists of V6 and V7. Meanwhile, the second group consists of variants V2, V3, and V5. The seven variant grouping pattern of *A. variabilis* based on RAPD markers has no similarity to the grouping pattern based on the results of glucomannan or calcium oxalate analysis.

Keywords: *A. variabilis*, Calcium oxalate, Glucomannan, RAPD, Variant.

INTRODUCTION

Amorphophallus variabilis is a native plant of Java, Madura, and Kangean Islands, Indonesia. This plant is located at 700 to 900m above sea level. *Amorphophallus variabilis* is locally called white *iles-iles* or *cocooan oray* in Sundanese. Local people are often confused by the terms *iles-iles* (yellow) for *A. muelleri* and *suweg* for *A. paeoniifolius* Dennst. Nicolson. Morphologically, *A. variabilis* does not have an aerial bulb in rachis, unlike *A. muelleri* [1].

Amorphophallus variabilis is one of Java's traditional foods. *A. variabilis* bulbs contain about 50% glucomannan, so they suitable to be used as raw materials in the food industry. Since the 1960s, rice plants became popular in Indonesia, therefore the utilization of tuber crops, including *Amorphophallus* species, decreased [1]. Besides glucomannan, *Amorphophallus* also contain calcium oxalate, which is a dicarboxylic acid with two C atoms in each carboxylic group. Consumption of oxalate in large quantities can cause kidney stones [2]. In addition, Calcium oxalate is dangerous for human health because this substance is anti-nutrient, which affects the unavailability of calcium in the body. Also, it can cause mechanical abrasion in the digestive tract and various fine tubules found in the kidneys [3].

Bojonegoro Regency is one area at East Java that has a lot of *iles-iles* (*Amorphophallus variabilis*) plants. Bojonegoro is located at 112°25' - 112°09' East Longitude and 6°59' - 7°37' South Latitude. Topographically, Bojonegoro area has land with a relatively flat slope. Existing data of Bojonegoro Regency showed that 91.26% of land in this region has a slope between 0-15%. The surface area of Bojonegoro Regency is relatively low, which is located between 25-500 m above sea level. Based on the location of the topography, Bojonegoro is one of the suitable areas for *A.variabilis* habitat. *A.variabilis* grows well in the area with condition 100-700 m above sea level [4].

From field observations, we found morphological variations of *A. variabilis* in Bojonegoro, especially in the petiole, both color and pattern of its skin. Pattern variations of petiole skin (*Corak*) may affect glucomannan content [5]. As a member of the Araceae family, glucomannan content of *Amorphophallus sp* has varying levels depending on the species.

* Correspondence address:
Achmad Yogi Pambudi
Email : yogyaahmad5@gmail.com
Address : Dept. Biology, University of Brawijaya, Veteran Malang 65145.

Therefore, it was necessary to determine both glucomannan and calcium oxalate content among *A. variabilis* variants in Bojonegoro with the expectation to find candidate variant which has high glucomannan content as well as low calcium oxalate content.

The existence of petiole pattern's skin variation from *A. variabilis* reflects genetic diversity. However, the morphology pattern sometime reflect its ecotype, so it is difficult to know the true diversity within a species. Therefore, we used molecular markers to determine the genetic diversity of plants that have a close genetic relationship [6]. Molecular markers have been used to study variation even among the species members of Araceae, such as molecular analysis, with RAPD markers on the *Amorphophallus* genus. *Amorphophallus* species that have been successfully analyzed by RAPD markers are *A. titanium* [7]. In this study, molecular analysis was performed with RAPD markers to determine whether the grouping of *A.variabilis* based on RAPD markers was similar to grouping based on glucomannan content or calcium oxalate content.

MATHERIAL AND METHOD

Preparation of Glucomannan Analysis

Amorphophallus variabilis tubers used in this research were taken from Bojonegoro, East Java. Glucomannan analysis followed the Chua Method [8], using 3.5 DNS reagents. This reagent will give a yellow color that is proportional to the amount of glucomannan. 3,5-Dinitro Salisilic Acid reagent is made by mixing solution A and solution B. Solution A was prepared by mixing phenol (0.7g), 10% NaOH (1.5 mL), aquadest (5 mL), and sodium bisulphite (0.7 g). Solution B was prepared by mixing potassium sodium tartrate (22.5 g), 10% NaOH (30mL), and 1% dinitro salicylic acid (88 mL).

Extract and Hydrolyzate of Glucomannan

Glucomannan extract is made by dissolving 0.2 g grind tuber-flesh in 100 mL of buffer solution (formic acid-sodium hydroxide), stirred for 4 hours then centrifuged at 4000 rpm for 20 minutes at 25°C; the white liquid produced is glucomannan extract. Glucomannan hydrolyzate was prepared by mixing 5 mL of glucomannan extract with 2.5 mL of 3M Sulfuric Acid, heated in boiling water bath for 1.5 hours, then add 2.5 mL of 6M NaOH and dilute to 25 mL using aquadest. A total of 2 mL of glucomannan extract or glucomannan hydrolyzate added with 1.5 mL of 3.5 DNS reagents, vortexes to homogenize. The absorbance of the solution was measured using a spectrophotometer with a wavelength of 550 nm. Glucomannan content determined according to the following formula.

$$glucomanan\ content\ (\%) = \frac{\varepsilon\ (5T - T0) x50}{m\ x\ (1-W)\ x\ 100} x100$$

Description:

ε : The ratio of glucose molecular weight and mannan residues in glucomannan to glucose and mannan molecular weights produced after hydrolysis, ε = 0.9
T : The amount (mg) of glucose in glucomannan hydrolyzate obtained from a standard curve
T_0 : The amount (mg) of glucose in glucomannan extract obtained from the standard curve
m : Sample mass (g)
W : Moisture content of the sample

Calcium oxalate

Calcium oxalate content determined through three stages, **1) Digestion**, at this stage, 2 g grind-tuber mixed with 190 mL of distilled water in a 250 mL measuring flask, 10 mL of 6M HCl was added, and the suspension digested at 100^0C for 1 hour, then cooled and added distilled water to 250 mL before filtration. **2) Precipitation**, the oxalate of the filtrate from every 125 mL of the suspension was added 4 drops of the red methyl indicator, then added NH4OH to obtain PH 4-4.5. Each part was heated at 90°C, then cooled and filtered to remove iron-sulfur ions. The filtration product reheated at 90°C, when the solution was stirred with a stirrer, 10 mL CaCl 5% was added. After heating, then cooled overnight at 5°C. The solution then centrifuged with a speed of 2500 rpm for 5 minutes. Subsequently, supernatant removed and precipitation result dissolved in 10 mL of 20% H_2SO_4 solution. **3) Permanganate titration ($KMnO_4$)**, the resulting filtrate was made up to 300 mL with water. 125 mL of aliquot filtrate heated to a slight boil, then titrated with standard 0.1N KMnO4 standard until produced a faded- pink color. The color was maintained for 30 seconds [9]. The calcium oxalate content was calculated using the following formula.

$$Calcium\ oxalate\ (g) = \frac{V\ x\ N\ Oxalate\ x\ Mr\ Calcium\ Oxalate}{1000}$$

Description:

V : the titration volume or titer volume = volume of $KMNO_4$ (mL) used in the titration until equilibrium reached or the equivalent point reached.
N : stated that the normality of calcium oxalate, which has been standardized
Mr (calcium oxalate) was 128.

DNA Isolation and Random Amplified Polymorphic DNA (RAPD) Analyzis

DNA isolated with the Doyle and Doyle CTAB method [10]. Leaf of *A. variabilis* was taken fresh

ISSN. 2087-2852
E-ISSN. 2338-1655

and weighed 0.1-0.2 g, and crushed using mortal-pestle. Before grinding, the leaf sample poured with enough liquid nitrogen. The results were transferred into the Eppendorf tube (hereinafter referred to as Epitube) and added 700 µL CTAB buffer then vortex. Epitube was incubated at 65°C in a water bath for 10 minutes.

After the incubation time fulfilled, extraction performed by adding phenol (PCI) as much as 700 µL (vortex). Epitube which has received the addition of PCI, centrifuged at 13000 rpm for 10 minutes at 4°C. Then the supernatant transferred to a new Epitube and the CI was added to the same volume as the supernatant volume, vortexed. The extraction was continued with centrifuged 13000 rpm for 5 minutes at 4°C, the supernatant was discarded carefully so that only pellets remained. DNA (pellets) was added with 0.1 volume of ammonium acetate and 2.5 volumes of absolute ethanol (vortex). The epitube containing DNA was then incubated at -20°C overnight. The following day the solution was centrifuged at 12000 rpm for 15 minutes at 4°C. Pellets washed with 70% cold alcohol with centrifuged at 12000 rpm for 15 minutes at 4°C. The pellets were dissolved in 20 µL TE solution (1 mM Tris HCl pH 8, 0.1 mM) for RAPD analysis.

The genomic DNA of *A. variabilis* amplified using 5 RAPD primers (Table 2), which based on previous studies [11]. The reaction mixture of each PCR tube was 10 µL containing 3 µL ddH_2O, 1 µL primary RAPD 10 pmol, 5 µL 2x PCR Intron master mix, 1 µL DNA template 50 ng. PCR reaction was carried out for 45 cycles using a thermocycler (Takara). Initial denaturation was done at 94°C for 5 minutes, then followed by 45 cycles namely denaturation of 94°C for 1 minute, annealing 36°C for 1 minute, extension 72°C for 2 minutes. The final extension is 72°C for 4 minutes and cooling at 4°C for 30 minutes [7].

Table 1. RAPD primers used in research

No.	Primers Code	Nitrogen base sequence 5'-3'
1	OPA-11	CAATCGCCGT
3	OPC-04	CCGCATCTAC
4	OPU-06	ACCTTTGCGG
5	OPC-07	CACACTCCAG
8	OPN-18E	AAGGTGAGGTCA

Data Analyzis

Glucomannan or calcium oxalate data were analyzed by analysis of variance (ANOVA) using the SPSS program followed by the Tukey test with $p < 0.05$ significant. Before conducting the ANOVA test, the homogeneity test was carried out by the Levene test, and the normality of the data was carried out by the Kolmogorov-Smirnov test. Glucomannan or calcium oxalate data grouping performed using Hierarchy Clustering with SPSS program.

The DNA band profile resulting from PCR amplification using RAPD primers converted into binary data with two values, namely one (1) for the presence of DNA bands and zero (0) for the absence of DNA bands. Only the clear band counted. Binary data was used to generate binary data matrices using the Numerical Taxonomy and Multivariate System (NTSYS-pc) version 2.1. The binary data matrix then reduced to a genetic similarity matrix between *A. variabilis* using Jaccard's similarity coefficient. The genetic similarity matrix created using Similarity for Quantitative Data (SIMQUAL). Based on the genetic similarity matrix, Cluster Analysis performed using the Unweighted Pair-Group Method with Arithmetic Average (UPGMA) by the Sequential Agglomerative Hierarchical Nested Cluster Analysis (SAHN) sub-program. The results of UPGMA grouping analysis were made in a cladogram using a tree-display, which is a sub-program Graphics NTSYS-pc Program [12].

RESULT AND DISCUSSION

A. variabilis, which found in the Bojonegoro District, has variations in petiole skin color called *corak* of both color and shape. Petiole skin color variations of seven variants of *A. variabilis* that found included dark green, light green, brownish-green and blackish green, while the colors of the *corak* were white, brownish, greenish-white and black and white. The shape of the *corak* of the seven variants is round, oval, and elliptical (Table 2, Fig. 1). Variations that arise in the skin color of the petiole and the color of the *corak* thought to be due to a combination of pigment levels such as chlorophyll, β-carotene and anthocyanin [13].

Table 2. *A. variabilis* variation

Variant	Petiole color	Pattern color	Shape of corak*
V1	Dark green	White	Irregular round
V2	Dark green	White	Ellipse and oval
V3	Brownish green	Brownish	Small round and tight
V4	Light green	White	Ellipse and big enough
V5	Light green	Greenish white	Round irregular and tight
V6	Brownish green	Light green	Large round and not tight
V7	Blackish green	Black and white	Small round and tight

*pattern-architecture of skin surface

ISSN. 2087-2852
E-ISSN. 2338-1655

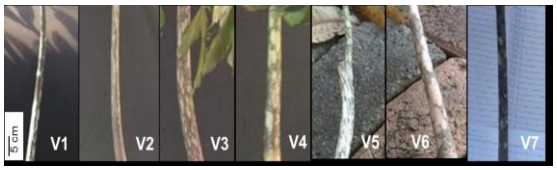


Figure 1. V1-V7: Variation of *A. variabilis*

Tubers are a means of vegetative propagation of plants and plant parts that used as storage for food reserves [14]. Tuber seeds used in this study were taken from the field with an average weight of 70.5-110.5 g. Then the tubers were planted until the aerial shoots part fall. After the aerial shoots fall, the tubers were harvested. Tuber yields of these plants weigh about 196.28-334 g, diameters range from 86.37-115.82 mm and tuber thickness ranges from 42.82-65.18 mm (Table 3).

Diameter and tuber weight of *A. variabilis* correlated very significantly with tuber glucomannan content. It meant that tuber diameter and tuber weight of harvest will influence the increase or decrease of tuber glucomannan content. The heavier tubers in *A. variabilis* may contain greater glucomannan [15]. In this study, however, heavy tubers and wide diameters, for example variant 1 (V1) or variant 2 (V2), did not contain high glucomannan (Table 3).

Table 3. Tuber weight, diameter, and thick of *A. variabilis* variant

Variant	Tuber weight (g)	Tuber diameter (mm)	Tuber thick (mm)
V1	304.60	115.82	48.03
V2	334.56	106.54	65.18
V3	286.24	99.12	55.47
V4	253.36	93.63	56.28
V5	196.28	86.37	42.82
V6	305.88	102.46	60.89
V7	234.65	92.24	59.25

Glucomannan is a type of special polysaccharides found in *Amorphophallus* tubers. The results of glucomannan extraction in seven variants of *A. variabilis* showed different glucomannan contents, ranging from 10-38%. The highest glucomannan content found in variant V3 of *A. variabilis* for 38% and the lowest in variant V6, which was 9% (Fig. 2). In summary, the glucomannan content of seven variants grouped into three categories, namely low, medium, and high categories. Low category comes from variants V2, V4, V5, and V6 (9-12%). Medium categories come from V1 and V7 (15-19%). High category consists of V3 (38%).

Mekkerdchoo *et al*. [16] used the same method (assay using 2,5-DNS) also grouped the glucomannan content of six *Amorphophallus* species (*A. muelleri, A. krausei, A. kachiensis, A. bulbifer, A. Xiei, and A. corrugatus*) into three groups: low (16%), moderate (22-24%), and high (32-42%). Whereas, Dini [5] grouped the glucomannan content of 8 variants of *A.variabilis* into low (16%), moderate (22-24%), and high (32-42) using a different method of centrifugation which was an adaptation of the Tatirat and Charoenrein method [17].

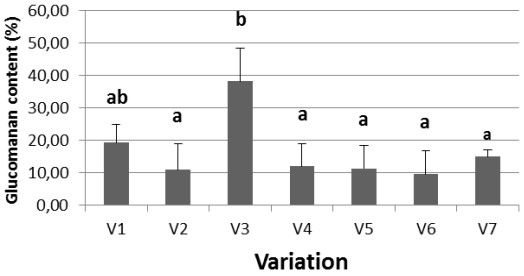


Figure 2. Glucomannan content in seven variants of *A. variabilis*, V1-V7: seven variants of *A. variabilis*. **Note:** the same notation shows no significant difference in the 0.05 Tukey test.

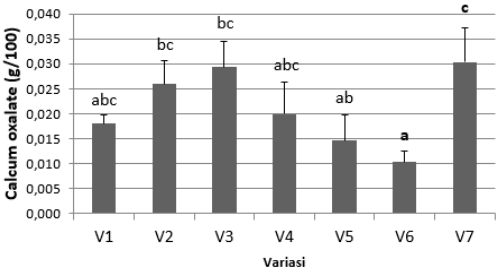


Figure 3. Calcium oxalate content in seven variants of A. *variabilis*, V1-V7: seven variants of A. *variabilis.* **Note:** the same notation shows no significant difference in the 0.05 Tukey test

The calcium oxalate content of seven variants of *A. variabilis* showed variants V1, V4, V5, and V6 have the same letter notation (Fig. 3). Meanwhile, the lowest V4 calcium oxalate content was significantly proven significantly different from V7, which had the highest oxalate content as indicated by different letter notation (c). The calcium oxalate content in *A. variabilis* variant V3 was not significantly different from variant V1 (b). Based on the picture above, it was known that the *A. variabilis* tuber contains different calcium oxalate content, ranging from 0.01 to 0.03 g. In these results, the V6 variant has the lowest oxalate content, while the V7 variant had the highest one, which was 0.03 g. These results showed that calcium oxalate was still safe for consumption (0.60-1.25 g per day) [18].

RAPD on seven variants of *Amophophallus variabilis* using OPA-11, OPC-04, OPU-06, OPC-07, and OPN-18E primers produced 24 amplicons

ISSN. 2087-2852
E-ISSN. 2338-1655

ranging from 500 bp to 4000 bp. Sixteen bands (61.54%) were polymorphic bands, and ten bands (38.47%) were monomorphic. A relatively high level of polymorphism with RAPD markers showed a high mark index. The highest number of polymorphic bands (4) was in the OPN-18E primer, while the lowest number (2) was in the OPU-06 primer (Table 6). The high band polymorphism in this study showed the high genetic diversity in variants of *A. variabilis*. Research by Poerba and Martanti [11] on *Amophophallus* muelleri using RAPD also showed genetic diversity. By using five primers, a total of 42 amplicons obtained, 29 bands (60.05%) were polymorphic, and 13 bands (30.95%) were monomorphic.

Table 6. RAPD amplification band

Primer	Polymorphic bands	Monomorphic bands	Total band
OPA-11	3	1	4
OPC-04	4	4	8
OPU-06	4	2	6
OPC-07	3	1	4
OPN-18E	4	0	4
Total	16 (61.54(%)	10 (38.46%)	26(100%)

Phenograms formed based on RAPD markers showed the formation of two groups of *A. variabilis*-variant. Group one has two subgroups. Subgroup one consists of variants V1 and V4 with a similarity coefficient 0.65, while subgroup two consists of V6 and V7 with a similarity coefficient 0.72. Group two consisted of V2, V3, and V5 variants with a 0.68 similarity coefficient (Fig. 4).

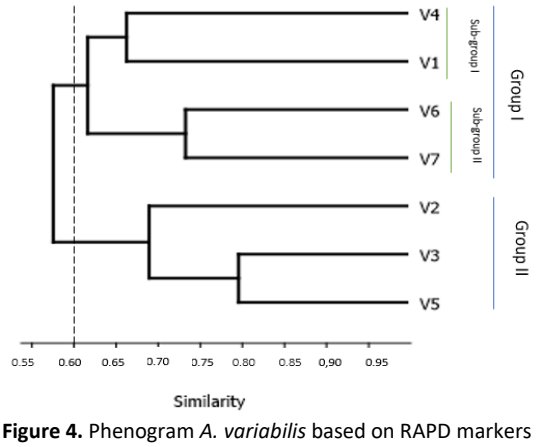


Figure 4. Phenogram *A. variabilis* based on RAPD markers

The results of grouping the seven variants of *A. variabilis* based on the RAPD markers did not show similarity in grouping patterns based on glucomannan or calcium oxalate content (Fig. 5 and 6). For example, in glucomannan, V3 and V7 formed one group, whereas, in phenogram based on RAPD markers, V3 grouped with V5, and V7 formed a group with V6. In calcium oxalate, V3 not only grouped with V7 but also with V2, V6, and V1. It can occur due to various factors, including the result of random amplification of primary RAPD that does not lead to a particular trait. One weakness of the RAPD marker for amplicon identity is unknown, unless the study is supported by pedigree analysis. From the results of the study, it can be said that the grouping based on RAPD markers cannot be used to predict groupings based on glucomannan or calcium oxalate content. Nevertheless, the results of grouping based on RAPD indicated that the seven variants of *A. variabilis* do indeed vary, the genomes were divided into two large groups.

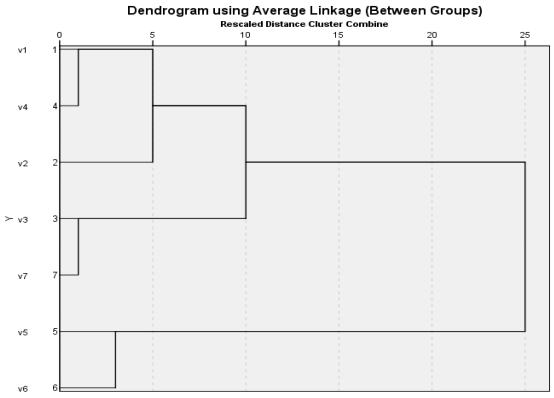


Figure 5. Glucomannan phenogram *A. variabilis* based on Hierarchy Clustering

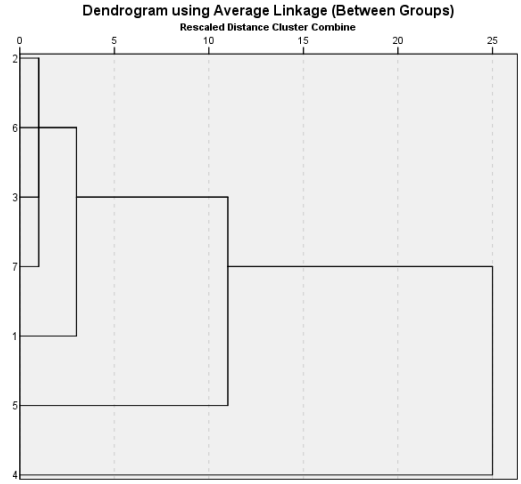


Figure 6. Calcium oxalate phenogram *A. variabilis* based on Hierarchy Clustering

CONCLUSION

The conclusion obtained from this study is that the glucomannan content in seven variants of *A. variabilis* ranges from 9-38%. Classification of variants based on glucomannan content is divided into three categories, namely low,

ISSN. 2087-2852
E-ISSN. 2338-1655

consisted of variants V2, V4, V5, and V6 with glucomannan content of 11-12%. The medium category consists of variants V1 and V7 with glucomannan content of 11, 12, and 15%. The high category consists of variants of V3 with 38% glucomannan content, while for the content of calcium oxalate contained in seven variants of *A. variabilis* ranged from 0.01 to 0.03 g. The variant with the lowest calcium oxalate is V6 with a value of 0.01 g, and the highest is the variant V7 with a value of 0.03 g. The range of calcium oxalate content is in a good range for health.

Phenograms formed based on RAPD markers showed the formation of two groups of *A. variabilis*. Group one has two subgroups, subgroups one consisted of variants V1 and V4, while subgroup two consists of V6 and V7. Group two consists of variants V2, V3, and V5. There is no similarity between the grouping of seven variants of *A. variabilis* based on variations in glucomannan or calcium oxalate content with grouping based on RAPD markers.

REFERENCES

[1] Santosa, E., N. Sugiyama, S. Hikosaka, T. Takano. 2012. Genetic variations of *Amorphophallus variabilis* Blume (Araceae) in Java using AFLP. *Jurnal Agron*omi *Indonesia.* 40(1). 62-68.

[2] Franceschi, V.R., P.A. Nakata. 2005. Calcium oxalate in plants: formation and function. *Annu. Rev.Plant Biol*. 56. 41-71.

[3] Brown, D. 2000. Aroids: plants of the arum family, 2nd Ed. Timber Press, Portland. Oregon.

[4] Yuzammi, A. Kurniawan, N.P.S. Asih, I. Erlinawati, W. Hetterscheid. 2017. *The Amorphophallus* of Indonesia. Centre for Plant Conservation-Botanic Gardens, LIPI.

[5] Dini, Z.Z. 2017. Variasi kandungan glukomanan dan hubungan kekerabatan menggunakan penanda RAPD pada berbagai varian *Amorphophallus Variabilis* Blume asal Tuban. Bachelor Thesis. Brawijaya University. Malang.

[6] Brown, E.J. 1991. Assesment of RAPD markers to detect genetic diversity in rice plants. *Crops Sci.* 3.670-675.

[7] Poerba, Y.S, Yuzammi. 2008. Pendugaan keragaman genetik *Amorphophallus titanium* Becc. berdasarkan marka Random Amplified Polymorphic DNA. *Biodiversitas*. 9(2). 103-107.

[8] Chua, M., K. Chan, T.J. Hocking, P.A. Williams, C.J. Perry, T. C. Baldwin. 2012. Methodologies for the extraction and analysis of konjac glucomannan from corms of *Amorphophallus konjac* K. Koch. *Carbohydr. Polym.* 87. 2202-2210.

[9] Iwuoha, C.I., F.A. Kalu. 1995. Calcium oxalate and physico-chemical properties of cocoyam (*Colocasia esculenta* and *Xanthosoma sagittifolium*) tuber flours as affected by processing. *Food Chem.* 54. 61-66.

[10] Doyle, J.J., J.L. Doyle. 1990. Isolation of plant DNA from fresh tissue. *Focus.* 12. 13-15.

[11] Poerba, Y.S., D. Martanti. 2008. Keragaman genetik berdasarkan marka Random Amplified Polymorphic DNA pada *Amorphophallus muelleri Blume* di Jawa. *Biodiversitas*. 9(4). 245-249.

[12] Rohlf, F.J. 2000. NTSYSpc Numerical Taxonomy and Multivariate Analysis system Version 2.1. Exter Publication. New York.

[13] Salisbury, F.B., C.W. Ross. 1992. Fisiologi tumbuhan, 2nd Ed. ITB. Bandung.

[14] Aksenova, N.P., L.I. Sergeeva, T.N. Konstantinova, S.A. Golyanovskaya, O.O. Kolachevskaya, G.A. Romanova. 2013. Regulation of potato tuber dormancy and sprouting. *Russian J. Plant Physiol*. 60. 301-312.

[15] Ambarwati, E., R.H. Murti. 2001. Analisis korelasi dan koefisien lintas sifat-sifat agronomi terhadap komposisi kimia umbi iles-iles *(Amorphophallus variabilis*). *Ilmu Pertanian*. 8(2). 55-61.

[16] Mekkerdchoo, O., C. Borompichaichartkul, A.L. Perrigo, G. Srzednicki, C. Prakitchaiwattana, A. Antonelli. 2016. Tracing the evolution and economic potential of Konjac Glucomannan in *Amorphophallus* species (*Araceae*) using molecular phylogeny and RAPD markers. *Phytotaxa*. 282(2). 81-106.

[17] Tatirat, O., S. Charoenrein. 2011. Physicochemical properties of konjac glucomannan extracted from konjac flour by a simple centrifugation process*. LWT - Food Sci. Technol.* 44(10). 2059-2063*.*

[18] Mawarni, R.T., S.B. Widjanarko. 2015. Penggilingan metode ball mill dengan pemurnian kimia terhadap penurunan oksalat tepung porang. *Jurnal Pangan dan Agroindustri.* 3(2). 571-581.

ISSN. 2087-2852
E-ISSN. 2338-1655

Repairing Cell Structure of Jejunum Tissue in RA-CFA Rat Model Improved by Caprine CSN1S2 Protein

Laili Nur Zaidah[1,2], Aris Soewondo[1], Fatchiyah Fatchiyah[1,2*]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia
[2]Research Center of Smart Molecule of Natural Genetics Resource, University of Brawijaya, Malang, Indonesia

Abstract

We aimed to analyze the effect of CSN1S2 protein in Etawah crossbred goat milk and yogurt on the histopathology of the jejunum and the amount of cell damage in Complete Freund's adjuvant (CFA)-induced rheumatoid arthritis (RA) rats. The rats were divided into six groups: the untreated control rats (C), control rats were given CSN1S2 protein from Etawah crossbred goat milk (CM) or yogurt (CY), RA rats, RA rats given CSN1S2 protein from Etawah crossbred goat milk (RAM) or yogurt (RAY). Hematoxylin-eosin staining was conducted for the histopathological analysis of jejunum. Statistical analysis was done using one-way ANOVA (a significance value of $p \leq 0.05$) followed by the Tukey test. Our study observed that the control, CM, and CY group have a normal histological structure of jejunum. The damage to the jejunum structure was reported in the RA group. The milk CSN1S2 protein was able to improve the structure of jejunum villi and increase the normal cell number in the jejunum of the RA group**,** similar to control. The RAY group showed an impaired jejunum structure and a high number of necrotic cells as in the RA group.

Keywords: Cell structure, CSN1S2, goat milk, jejunum, rheumatoid arthritis.

INTRODUCTION

The prevalence of Rheumatoid Arthritis (RA) sufferers in Indonesia in 2013 reached 45.59%. This value raised from the previous year, which was 29.35% in 2011 and 39.47% in 2012 [1]. The RA cases that continue to increase are related to aging, lifestyle changes, and nutritional deficiencies [2,3]. Previous research stated that RA is also associated with the inflammation of the intestine [4,5]. Inflammatory conditions are caused by an increase in autoreactive T cells to produce proinflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and TNF-α [6,7]. The autoreactivity of T cells is caused by an imbalance of normal intestinal microflora. This condition can be caused by the presence of adjuvant induction [5].

Previous studies reported that RA disease induced by Complete Freund's Adjuvant (CFA) caused damage to the villi structure of ileum [8]. The jejunum is located adjacent to the ileum. The mucosal epithelial cells and jejunal villi are susceptible to damage due to the food contaminants, the cell physiological conditions (fasting, mutagens, chemical drugs, and ionizing radiation), and the inflammation of the large intestine [9,10]. The villus in jejunum is predicted to be impaired due to the inflammation in RA. However, the correlation between RA and inflammation in jejunum has not been studied thoroughly.

The RA treatment so far is carried out by using synthetic drugs that are not only expensive but also have long-term side effects [10]. The alternative management of RA is by consuming adequate and balanced nutrition. Nutrition can be derived from vegetable or animal protein [11]. One of an animal protein source is milk. Cow milk and goat milk are rich in bioactive peptide contents from proteins such as α-casein-S1, α-casein-S2 (CSN1S2), β-casein, and κ-casein [12,13]. Previous research found a specific protein at 36 kDa molecular weight in Etawah crossbred goat milk and yogurt that identified as CSN1S2 protein [14]. Further research is needed to investigate whether the CSN1S2 protein of Etawah crossbred goat milk and yogurt improves the jejunum of CFA-induced RA rats.

METHOD

Experimental Animals

This study used 24 male Wistar rats (*Rattus norvegicus*) with 12 weeks of age and 150-200 g of body weight. The rats were obtained from the Integrated Research and Testing Laboratory (LPPT), Gadjah Mada University, Yogyakarta, Indonesia. For one week, the acclimatization of the animals was done in the Animal Laboratory, Biosciences Institute, Brawijaya University, Malang, Indonesia. They were exposed to a 12-hour light/dark cycle at room temperature with 50±5% relative humidity. They fed with a standard rat laboratory diet and drinking water. The rats were divided into six treatment groups (n = 4 for each group), namely the untreated

*Corresponding Address:
Prof. Fatchiyah, PhD.
Email : fatchiya@ub.ac.id
Address : Dept. Biology, University of Brawijaya, Veteran Malang, 65145.

ISSN. 2087-2852
E-ISSN. 2338-1655

control rats (C), control rats given CSN1S2 protein from Etawah crossbred goat milk (CM), control rats fed CSN1S2 protein from Etawah crossbred goat yogurt (CY), Rheumatoid Arthritis rats (RA), RA rats given CSN1S2 protein from Etawah crossbred goat milk (RAM), and RA rats fed CSN1S2 protein from Etawah crossbred goat yogurt (RAY).

The Establishment of CFA-induced Rheumatoid Arthritis Animal Model

Multiple doses of CFA injection established the RA rat model. The rats were injected by 100 µL of CFA (Sigma-Aldrich Inc., Saint Louis, Missouri, USA) subcutaneously using a 1 mL syringe. After 14 days, the 50 µL of CFA was injected into the rats' intradermal. The CSN1S2 protein of Etawah crossbred goat milk and yogurt was administered to the rats 24 hours after the last CFA injection [8].

Isolation of Caprine CSN1S2 Protein

The Etawah crossbred goat milkwas obtained from the Singosari Regional Technical Implementation Unit (UPT) of Livestock in Malang, East Java, Indonesia. The milk was fermented into yogurt according to the previous research [14] with some modifications. The CSN1S2 protein from yogurt and milk was isolated according to the previous study [15]. We measured the protein concentration using the NanoDrop spectrophotometer and stored the protein at -20°C. The treatment of CSN1S2 protein of milk and yogurt into the rats was carried out orally for three months. The dose of CSN1S2 protein used in this study was $2mg.kg^{-1}$ of body weight. This research has been approved by the Research Ethics Committeeof Brawijaya University, Malang, and East Java, Indonesia (Certificate number, KEP-90-UB).

Histopathological Analysis

All rats were sacrificedto collect the jejunum. The jejunum of each rat was washed in phosphate-buffered saline (PBS) pH 7.4. We did the fixation of jejunum in 4% paraformaldehyde. The organs were dehydrated using graded alcohol concentration (30%, 40%, 50%, 60%, 70%, 80%, 90%, 96%) followed by clearing using xylene. We infiltrated our specimens with liquid paraffin at 62°C for 4 hours. The specimens were embedded with liquid paraffin and cooled overnight. A rotary microtome performed the serial sectioning of jejunum with 5µm of thickness. The histological slides were placed on a hot plate with atemperature of 42°C at least for 24 hours. Deparaffinization of tissue slides was done using xylene, followed by dehydration using a graded series of alcohol (absolute ethanol, 90%, 80%, 70%). The slides were washed using running water for 15 minutes. After that, the slides were stained with hematoxylin-eosin (HE). After staining with eosin, we washed the slides using running water and dehydrated them with 70% ethanol and 90% for 2 minutes, respectively, then continued with absolute ethanol. The sections were embedded in Entellan (Merck, Darmstadt, Germany) and covered with a cover glass. The morphology of normal cells, pyknosis, karyorrhexis, and karyolysis cells were observed using an Olympus BX 53 microscope with cellSens Standard software version 1.5 (Olympus Corporation, Tokyo, Japan) [15].

Data Analysis

Data analysis was performed by comparing the number of normal cells and necrotic cells statistically by using SPSS software version 16.0 for windows (SPSS Inc., Chicago). The results were expressed by means ± standard deviation. We performed the statistical analysis using one-way ANOVA test followed by the Tukey test. The significance level used in this study was $P<0.05$.

RESULTS

The histopathology of rat jejunum for each treatment group is shown in Figure 1. The mucosal layer in the jejunum of control rats has a normalvillous shape. Villi are composed of columnar epithelial cells and lined with a striated border from enterocyte. In the RA group, we observed an epithelial cell exfoliation in the mucosal layer (desquamation). In addition, the infiltration of inflammatory cells to the lamina propria of villi, the reduction in the depth of the crypts, and a decrease in the distance between the muscularis mucosae to the muscularis externa have occurred in them.

The treatment of CSN1S2 protein from Etawah crossbred goat milk or yogurt into the control rats resulted in a similar jejunum tissue structure as in the control group. Interestingly, the RA rats that were given the goat milk CSN1S2 protein showed an improvement in the villous epithelial structure and an increase in the depth of the crypts. On the contrary, the mucosal layer of yogurt CSN1S2 protein-treated RA rats underwent an exfoliation, and a decrease in the crypts depth. There was also a decrease distance between muscularis mucosae and muscularis externa in the RAY group as RA group. Moreover, their villi were blunt, and adhesion occurred in them.

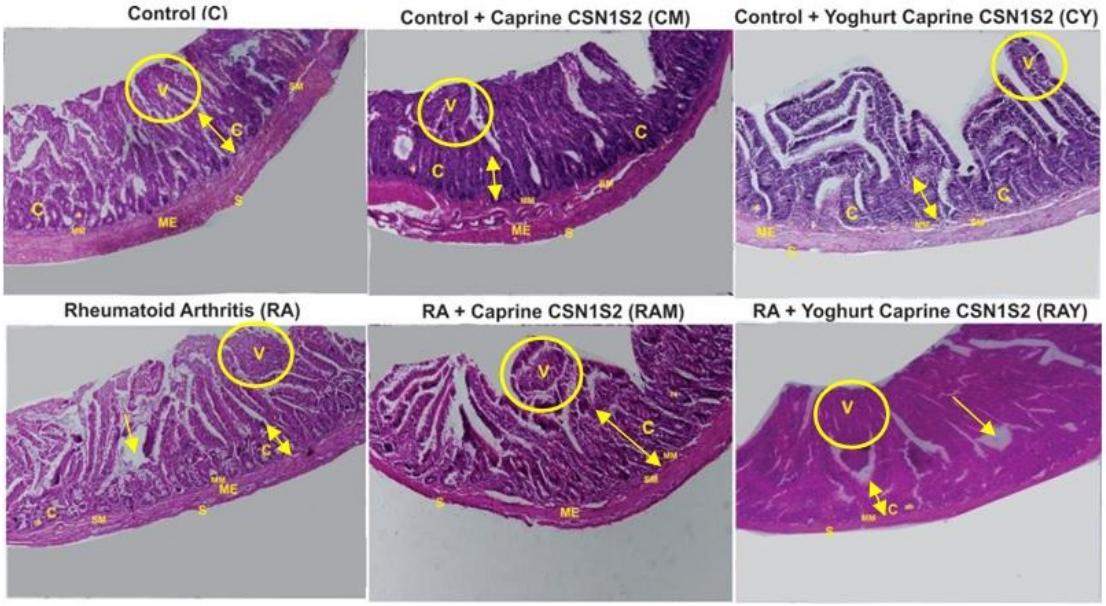


Figure1. Histopathological profile of jejunal tissues in the control and RA-CFA rat model using H&E staining (100X magnification). **Description:** C– crypts; ME–muscularis externa; MM–muscularis mucosae; SB–striated border of enterocyte; S–serosa; SM–submucosa; V–villi; ↔–depth of crypts; →– exfoliation of epithelial cells (desquamation); O–change in villi shape.

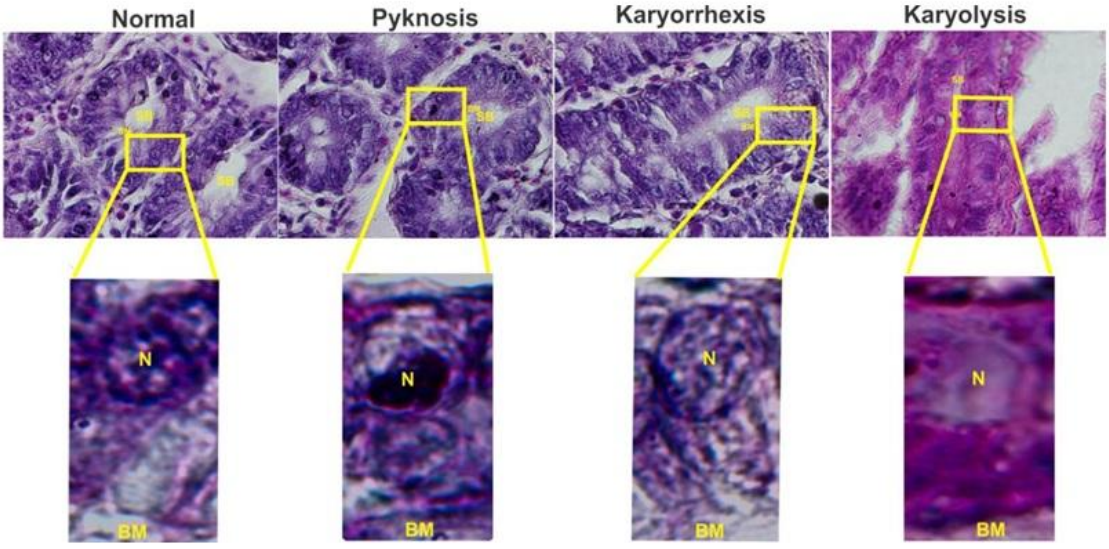


Figure 2. The histology of rat jejunal tissue shows the normal cells and necrotic cells (pyknosis, karyorrhexis, karyolysis) using H&E staining (1000X magnification). **Description:** BM–basement membrane; N–Nuclear; SB–striated border of enterocyte.

Table1. The number of normal and necrotic cells in the control and RA-CFA rats model with and without treatment of Caprine CSN1S2 protein from fresh milk and yogurt.

Parameter	C	CM	CY	RA	RAM	RAY
Normal	33.02 ± 7.22^{c}	32.78 ± 9.07^{c}	27.55 ± 7.15^{bc}	9.93 ± 0.70^{a}	29.33 ± 2.01^{bc}	13.73 ± 2.32^{ab}
Pyknosis	2.83 ± 0.49^{a}	5.53 ± 0.83^{ab}	6.87 ± 0.99^{b}	11.53 ± 0.99^{c}	4.33 ± 1.45^{ab}	6.02 ± 1.73^{b}
Karyorrhexis	2.22 ± 1.02^{a}	1.00 ± 1.73^{a}	2.50 ± 2.18^{a}	3.11 ± 0.19^{a}	1.50 ± 1.50^{a}	4.31 ± 1.68^{a}
Karyolysis	3.00 ±1.11^{a}	6.47 ±2.00^{a}	4.53 ±0.42^{a}	18.27 ± 2.66^{b}	4.96 ± 1.18^{a}	15.47 ± 2.32^{b}
Necrosis	8.06 ± 1.45^{a}	13.00 ± 2.40^{a}	13.90 ± 2.50^{a}	32.91 ± 3.74^{b}	10.79 ± 1.69^{a}	25.79 ± 4.77^{b}

Notes: Values are presented as mean ± standard deviation. C: control rats group, CM: control rats group + CSN1S2 protein from Etawah crossbred goat milk, CY: control rats group + CSN1S2 protein from Etawah crossbred goat yogurt, RA: Rheumatoid Arthritis rats group, RAM: RA rats group + CSN1S2 protein from Etawah crossbred goat milk, RAY: RA rats group + CSN1S2 protein from Etawah crossbred goat yogurt.

ISSN. 2087-2852
E-ISSN. 2338-1655

The cell damage can be evaluated by comparing the numberof normal cells versus necrotic cells (Fig. 2). Statistical analysis showed that the average number of normal cells in the milk CSN1S2 protein-treated control rats (32.78 ± 9.07) and in the yogurt CSN1S2 protein-treated control rats (27.55 ± 7.15) were not significantly different from the control group (33.02 ± 7.22) (Table 1). The RA group (9.93 ± 0.70) experienced a decrease in the normal cell number compared with the control group. Compared with the RA group, the administration of CSN1S2 protein from milk or yogurt gave rise to the RA rats' normal cell number.

Our study reported that the average number of pyknosis cells in the RA group was the highestand significantly different from other groups. The CSN1S2 protein from milk or yogurt treatment reduced the pyknosis cell number in RA rats. However, the treatmentgave rise to the elevated pyknosis cell number in control rats.

The average number of cells undergoing karyorrhexis between all treatments did not differ significantly. RA rats had the highest number of karyolysis cells. The CSN1S2 protein of milk was capable of lowering the karyolysis cell number in RA rats. The control group, CM group, and CY group hadlower necrotic cell damage than the RA and RAY group. The administration of CSN1S2 protein of Etawah crossbred goat milk into the RA rats affected the necrotic cells number to decrease (10.79 ± 1.69) compared with the RA group.

DISCUSSION

The presence of CFA causes damage to the epithelial structure. CFA is composed of attenuated pathogenic bacteria, such as Enterobacter (*Mycobacterium butyricum*). Furthermore, pathogenic bacteria will release endotoxin compounds such as Lipopolysaccharide (LPS) [16]. CFA can cause dysbiosis, which is an imbalance between nonpathogenic and pathogenic bacteria, resulting in the dominance of pathogenic bacteria. Pathogenic bacteria can increase intestinal permeability [5]. Permeability may increase due to the decline of Tight junctions (TJs) that maintain the epithelium structure and shape. Epithelial cells in the intestinal mucosa play a role in improving barrier function. Changes in the epithelial cells of villi may interfere with lipid absorption in lymphatic or lacteal capillaries [17]. The blunt villi occurred in the RAY group was due to the accumulation of toxins in enterocytes from pathogenic bacteria, which resulted in the loss of electrolyte fluid and mucous secretion. Therefore, the villi became shorter, and cellular desquamation occurred that caused the absorption of nutrients disturbed [18].

RA and RAY groups had a decrease distance between the muscularis mucosae and the muscularis externa and a reduction in the depth of the crypts. The muscularis mucosa functions in the absorption and secretion of nutrients and villous movements. The decrease of the distance between the muscularis mucosa and can reduce villous movement so that the absorption of nutrients is impaired [19]. A reduction in the depth of the crypts can lower the anti-microbial mucus and lysozyme secretions.As a result, an invasion of bacterial pathogens may take place inducing the paneth cells to secrete tumor necrosis factor-alpha as an inflammatory mediator [17].

The CSN1S2 protein of Etawah crossbred goat milk can reduce the damage to the jejunum tissues of the RA group due to its properties as an anti-oxidative [20], immunomodulatory [21], anti-microbial [22] and anti-inflammatory compounds in tissues [8]. Besides, CSN1S2 protein can also improve the perforation in the ileum due to the accumulation of toxins in the intestine [23]. However, theCSN1S2 protein of Etawah crossbred goat yogurt was not effective in repairing the jejunum. Thatis consistent with the previous research stating that CSN1S2 protein from Etawah goat yogurt can not control normal flora in the intestine. It causes blunt villi in the ileum as well [8].

Rheumatoid arthritis is a gastrointestinal disease caused by an imbalance of intestinal microbiota. This condition is related to the ability of adjuvants to change T cell responses and stimulate systemic inflammation. Adjuvants can increase intestinal permeability and facilitate antigen invasion in the lumen through the immune system in the intestinal mucosa [5]. The pathogenesis of RA disease is characterizedas an increase in cell damage and inflammatory conditions in the ileum due to the imbalance of intestinal homeostasis [8]. Pathological conditions are caused by the paneth cells secreting tumor necrosis factor-alpha as an inflammatory mediator [17]. Inflammation can cause necrosis [24]. This study revealed that the necrotic cell number in the RA group was the highest and significantly different from the other groups. The yogurt given to the control group is

safe to consume since its number of normal and necrotic cells did not differ significantly from the control and CM group. Our study reported that CSN1S2 protein from Etawah crossbred goat milk could trigger the normal cell proliferation indicated by the high number of the cells. But, if the goat milk is fermented into yogurt, it cannot increase the normal cell number.

The SEM analysis of bone in the previous study [25] showed that the CSN1S2 protein from Etawah crossbred goat yogurt could increase phosphorus minerals but low in calcium and nickel. The increase in phosphorus enhances ATP degradation, which results in elevated inorganic phosphate in cells so that the tissue experiences hypoxia. The other study reported that Caprine CSN1S2 protein milk could improve the proliferation of normal cells and reduce the necrotic cell number in the RA group due to the physiological role of CSN1S2 protein as an anti-oxidative [20] and immunomodulators [21,26]. Moreover, bioactive peptides of CSN1S2 protein of Etawah crossbred goat milk can increase the proliferation of pre-osteoblast cells to control cell inflammation due to oxidative stress [25]. The yogurt from Etawah goat milk is not suitable for routine consumption by RA patients because it can result in decreased normal cells number and elevated necrotic cells. Thought, we take the recommendation that the fresh milk Etawah crossbred goat to be consumed by patients with RA as proper. However, the Etawah crossbred goat yogurt is not recommended for routine consumption due to the effect may worsen rheumatoid arthritis patients.

CONCLUSION

This study concluded that the damaged cells of jejunum villi are successfully repaired by CSN1S2 protein in fresh milk Etawah crossbred goat milk. However, the yogurt CSN1S2 protein failed the cell improvements.

Acknowledgments

This study was supported by the Professor research Grant 2019 (DIPA-042.01.2.400919/ 2019), Brawijaya University. We thank Biosains Institute, Brawijaya University, for providing the laboratory facilities. We gratefully acknowledge Pupimadita Tizar Afdora, M.Si for laboratory assistance, and Hazna Noor Meidinna, S.Si, for English proofreading.

REFERENCES

[1] Fundamental Health Research (Riset Kesehatan Dasar Riskesdas). 2013. Ofice of Health Research and Development (Badan Penelitian dan Pengembangan Kesehatan). Indonesian Ministry of Health. Available at: http://www.depkes.go.id/resources/download/general/hasilRiskesdas2013.pdf.

[2] Shlotzhauer, T.L. 2014. Living with rheumatoid arthritis. Johns Hopkins University Press. Maryland.

[3] Silva, G.B., B.Z. Reis, S.M.F. Cozzolino. 2016. Micronutrients Deficiencies in Rheumatoid Arthritis Patients. *Int. J. Pathol. Clin. Res*. 2(29). 1-5.

[4] Actis, G.C. 2014. The gut microbiome. *Inflamm. Allergy Drug. Targets.* 13(4). 217-23.

[5] Yeoh, N., J.P. Burton, P. Suppiah, G. Reid, S. Stebbings. 2013. The Role of the Microbiome in Rheumatic Diseases. *Curr. Rheumatol. Rep.* 15(314). 1-11.

[6] Jethwa, H., S. Abraham. 2017. The evidence for microbiome manipulation in inflammatory arthritis. *Rheumatology*. 56(9). 1452-1460.

[7] Hochberg, M.C., A.J. Silman, J.S. Smolen, M.E. Weinblatt. 2014. Rheumatology, 6th Ed. Mosby, Elsevier Ltd. Missouri.

[8] Rohmah, R.N., E. Widjajanto, F. Fatchiyah. 2015. Protective effect of CSN1S2 protein of goat milk on ileum microstructure and inflammation in rat-CFA-induced rheumatoid arthritis. *Asian Pac. J. Trop. Dis.* 5(7). 564-568.

[9] Inagaki-Tachibana, E., T. Tsukahara, K. Kaji, R. Eguchi, H. Kanazawa, H. Hayashi., Y. Suzuki. 2009. Involvement of DNA fragmentation of enterocytes in mucosal injury to a mouse jejunum incubated in using chambers. *Nagoya J. Med. Sci.* 71(1-2). 11-18.

[10] Zheng, Z., Z. Zuo, P. Zhu, F. Wang, H. Yin, X. Peng, J. Fang, H. Cui, C. Gao, H. Song, P. Ouyang, Y. Zhou, S. Zhao. 2017. Research Paper A study on the expression of apoptotic molecules related to death receptor and endoplasmic reticulum pathways in the jejunum of AFB_1-intoxicated chickens. *Oncotarget.* 8(52). 89655-89664.

[11] Vitetta, L., S. Coulson, J. Schloss, S.L. Beck, R. Allen, A. Sali. 2012. Dietary recommendations for patients with rheumatoid arthritis: a review. *Nutr. Diet. Suppl.* 4. 1-15.

[12] Greppi, G.F., P. Roncada, R. Fortin. 2008. Protein components of goat's milk. In:

Pulina, G., A. Cannas (Eds). Dairy Goats Feeding and Nutrition, 2nd Ed. Bologna, CAB International. Oxfordshire. 71-94.

[13] Cozma, A., S. Andrei, D. Miere, L. Filip, F. Loghin. 2011. Proteins profile in milk from three species of ruminants. *Not. Sci. Biol.* 3(1). 26-29.

[14] Budiarti, I.K., M. Padaga, F. Fatchiyah. 2013. Nutritional composition and protein profile of goat yogurt PE with double culture between *Streptococcus thermophilus* and *Lactobacillus* Species. *Cukurova Medical Journal*. 38(4). 681-686.

[15] Bia, R.R., R.P. Virgirinia, B. Setiawan, A. Soewondo, F. Fatchiyah. 2015. Goat milk CSN1S2 is able to decrease the severity scoring, TNF-a, and RAGE expression in complete Freund's adjuvant-induced rheumatoid arthritis model of rats. *BGM*. 7. 64-71.

[16] Nagai, N., T. Tanino, Y. Ito. 2015. Excessive interleukin 18 relate the aggravation of indomethacin-induced intestinal ulcerogenic lesions in Adjuvant-Induced Arthritis Rat. *Biol. Pharm. Bull.* 38(10). 1580–1590.

[17] Tlaskalová-Hogenová, H., R. Stěpánková, H. Kozáková, T. Hudcovic, L. Vannucci, L. Tučková, P. Rossmann, T. Hrnčíř, M. Kverka, Z. Zákostelská, K. Klimešová, J. Přibylová, J. Bártová, D. Sanchez, P. Fundová, D. Borovská, D. Srůtková, Z. Zídek, M. Schwarzer, P. Drastich, D.P. Funda. 2011. The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases. *Cell. Mol. Immunol.* 8(2). 110–120.

[18] Freedman, J.C., M.A. Navarro, E. Morrell, J. Beingesser, A. Shrestha, B.A. McClane, F.A. Uzal. 2018. Evidence that Clostridium perfringens Enterotoxin-induced Intestinal Damage and Enterotoxemic Death in Mice Can Occur Independently of Intestinal Caspase-3 Activation. *Infect. Immun*. 86(7). e00931-17.

[19] Peckham, M. 2011. Histology at a glance. John Wiley and Sons Ltd. Chichester.

[20] Chotimah, C., G. Ciptadi, B. Setiawan, F. Fatchiyah. 2015. CSN1S2 protein of goat milk inhibits the decrease of viability and increases the proliferation of MC3T3E1 pre-osteoblast cell in methylglyoxal exposure. *Asian Pac. J. Trop. Dis.* 5(3). 219-223.

[21] Sharma, S., R. Singh, S. Rana. 2011. Bioactive peptides: a review. *Int. J. Bioautomation.* 15(4). 223-250.

[22] Sujono, Y. Bekti, A. Hikmawan, A. Saga, Yuananda. 2016. Use of milk goat yoghurt lowering uric acid, cholesterol and blood glucose. The 2nd International Conference on Science, Technology, and Humanity. ISSN: 2477-3328.

[23]. Agustina, V., B. Setiawan, F. Fatchiyah. 2015. Acute toxicity of caprine alpha S2-casein protein on the microstructures and mineral profiles of rat ileum. *Asian Pac. J. Trop. Dis.* 5(9). 721-725.

[24] Elmore, S.A., D. Dixon., J.R. Hailey, T. Harada, R.A. Herbert, R.R. Maronpot, T. Nolte, J.E. Rehg, S. Rittinghausen, T.J. Rosol, H. Satoh., J.D. Vidal., C.L. Willard-Mack., D.M. Creasy. 2016. Recommendations from the INHAND Apoptosis/Necrosis Working Group. *Toxicol. Pathol.* 44(2). 173-188.

[25] Fatchiyah, F., B. Setiawan, S. Suharjono, Z. Noor. 2015. The anti-osteoporosis effects of CSN1S2 protein of goat milk and yoghurt on a complete Freund's adjuvant-induced Rheumatoid Arthritis model in rats. *BGM*. 7. 139-146.

[26] Fatchiyah, F., S.J. Raharjo, F.R.P Dewi. 2015. Virtual Selectivity Peptides of CSN1S2 Protein of Local Goat Etawah Breeds Milk Modulate Biological Mechanism of Calmodulin. *Int. J. Pharm. Bio. Sci*. 6(2). 707 -718.

The Effectiveness of Combination of *Momordica charantia* Extract and *Averrhoa bilimbi* on Nf-Kb Activation in Mice (*Mus musculus*) Balb/C Hyperglycemia Models

Intan Sartika Risky Sampurna[1], Muhaimin Rifa'I[2*], Sri Rahayu[2]

[1]Magister Biology, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia

[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia

Abstract

Hyperglycemia is a medical condition when the blood glucose levels increase exceeds the reasonable limit. Hyperglycemia is a typical sign of diabetes mellitus (DM). Indonesia is the sixth-ranked country in the world after China, India, United States, Brazil, and Mexico. Treatment with herbs is currently being developed. *Pare* (*Momordica charantia*) and *Belimbing wuluh* (*Averrhoa bilimbi*) are plants that found around us. Some studies state that each of these plants can be anti-diabetic. The hyperglycemia can cause an immune system disorder characterized by pancreatic β cell death involving IL-1β, kappa B (NF)-κB nuclear factor, and Fas. The ability of NF-kB activation will affect the number of cytokines expressed by T cells, namely TNF-α, and IFN-γ. The purpose of this study is to determine the effect of NF-kB activation on blood glucose levels in hyperglycemia mice. The results showed that the positive control treatment showed an increase in the number of NF-kB activations in CD4 and CD8 cells. EPBW (combination of A*verrhoa bilimbi* extract and *Momordica charantia*) administration results at a dose of 10 $mg.kg^{-1}$ BW showed a reduction in the amount of activated NF-kB as a substitute for the reduction. In addition, that dose can reduce blood sugar levels in mice hyperglycemia model.

Keyword: *Averrhoa bilimbi,* Hyperglycemia, *Mamordica charantia,* NF-kB.

INTRODUCTION

Hyperglycemia is a typical sign of diabetes mellitus (DM). Based on WHO data, 422 million people live with diabetes [1]. Diabetes is one of the autoimmune diseases caused by decreased insulin secretion by pancreatic β cells [2]. The hyperglycemia will cause an immune system disorder characterized by pancreatic β cell death. This cell death involves IL-1β and nuclear factor kappa B (NF-kB). The ability of NF-kB activation will affect the number of cytokines expressed by T cells, such as TNF-α [3].

Herbal treatment can reduce the risk of dependence, such as treatment using chemicals. *Averrhoa bilimbi* is a plant we can found around the people of Indonesia. *Averrhoa bilimbi* has several active substances, including antioxidants as anti-bacteria, anti-diabetes, and anti-inflammatory [4].

Momordica charantia contains charantin, which can recover pancreatic beta cells. In addition, *A. bilimbi* as anti-hyperglycemic whereby giving ethanol extracts of *A. bilimbi* can affect the blood glucose levels of mice, that have hyperglycemia. Flavonoids are believed to be antidiabetic substances as inhibitors of the alpha-glucosidase enzyme that delay the absorption of carbohydrates so that the blood glucose levels will decrease [5]. Combining the two plants is expected to increase the effectiveness of active substances contained in the two plants. The purpose of this study is to determine the effect of EPBW on NF-kB activation and blood glucose level in mice with hyperglycemia model.

MATERIAL AND METHOD

This research was conducted from November 2019 to February 2020 in the Animal Laboratory of Malang State University, and the Laboratory of Physiology, Structure and Animal Development, Department of Biology, Universitas Brawijaya, Malang, East Java, Indonesia.

Description of Tested Animals

Tested animals used in this study were 25 Bab/c mice with 6-week-old female sex obtained from the Animal Physiology Laboratory of Islamic University of Malang. The average weight of mice was 23 g.

Research Design

This study used a Completely Randomized Design (CRD) with five treatment groups with five replications for each treatment. The treatments of this study were healthy control, pain control, and treatment with three different EPBW doses. Tested animals in the control group and treatment group were given streptozotocin (STZ)

* Correspondence address:
Muhaimin Rifa'i
Email : Immunobiology@ub.ac.id
Address : Dept. Biology, University of Brawijaya, Veteran Malang, 65145

injections with a single dose of 145 mg.kg^{-1} BW dissolved in an STZ buffer with a pH of 4.5. After three days of the injection, fasting blood sugar from mice was measured. Mice for the treatment group were mice, which had blood glucose <200 mg.dL^{-1}. The treatment group used three doses of combinations of EPBW in 10, 40, 160 mg.kg^{-1} BW. Research design referred to the approval of the ethics commission number 1109-KEP-UB.

Preparing EPBW

The extract of *A. bilimbi* and *M. Charantia* were obtained from Materia Medika in Malang, East Java. The solvents used were ethanol 96%, which is a solvent that has a group of hydroxyl, which is polar and group alkyl, which is non-polar so it can dissolve the entire secondary metabolites compound that contained in the plants [6]. The compound from bitter melon is a compound of three secondary metabolites that is polar or non-polar, so the organic solvent, such as ethanol 96%, is needed. Ethanol extract can activate the flavonoid, saponin. By doing the extraction, crude extracts were obtained from the two plants [5]. Making EPBW were performed using a solvent of distilled water of 150 mL for each dose treatment. Making extracts were done every three days. The EPBW combination was given using a sonde adjusted to the weight of the mouse and carried out every day for 15 days.

Cell Isolation of Spleen

Mice that had been treated for 14 days were surgically removed to isolate cells from spleen organs by being crushed in PBS solution, then transferred in a 15 mL propylene tube. After all the spleens from the experimental animals were collected, centrifugation was carried out with a speed of 2500 rpm and a temperature of 10°C for 5 min. The supernatant from the centrifugation was removed, and pellets added with 1 mL PBS and homogenized.

Antibody Staining and Flow-cytometric Analysis

Flow-cytometric analysis performed using DB Cellquest Pro software. Intracellular cytokine staining was performed by a Cytoperm/Cytofix kit (BDBiosciences Pharmingen) according to the manufacturer protocol and modified by Rifa'i [7]. Staining that was used was FITC-conjugated rat antimouse CD4, PE-conjugated rat-antimouse CD25 and PE/Cy5 conjugated rat antimouse NF-kB, FITC-conjugated rat antimouse CD4, PE-conjugated rat- antimouse TNF-α and PE/Cy5 conjugated rat anti-mouse IFN-γ.

Statistical Analysis

Data analysis using BDCellQuest Pro software with testing with statistical analysis using *one way* ANOVA (*Analysis Of Variance*) using SPSS version 25. The results of the study were said to be significant if the results obtained were p <0.05. If the results of the study are significant, further tests are conducted using Tukey Test.

RESULT AND DISCUSSION

EPBW Decrease The Activation of NF-kB in CD4 Cells

The positive control group is a hyperglycemia (K+) mouse model of the relative number of CD4+ cells that express the highest NF-kB compared to the healthy control group (K-), which is 7.45% (p <0.05). The average healthy control was lower than the pain control that was 6.3% but significantly different from the average relative number of cells expressing NF-kB in the hyperglycemia mouse group P1 5.5%, but not different in the P2 group by 6.3% and P3 of 6.9% as indicated by the notation in Figure 1.

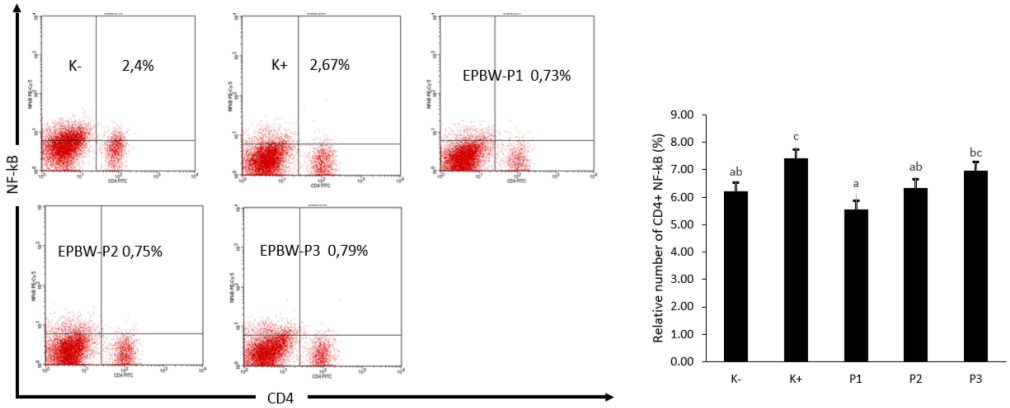


Figure 1. Graph of the Decreasing of NF-κB activation in CD4 cells in hyperglycemia mice. **Note:** K- = healthy control, K+ = pain control, P1 = hyperglycemia mice model with a treatment dose of 10 mg.kg^{-1} BW, P2 = hyperglycemia mice model with a treatment dose of 40 mg.kg^{-1} BW, P3 = hyperglycemia mice model with a treatment dose of 160 mg.kg^{-1} BW.

ISSN. 2087-2852
E-ISSN. 2338-1655

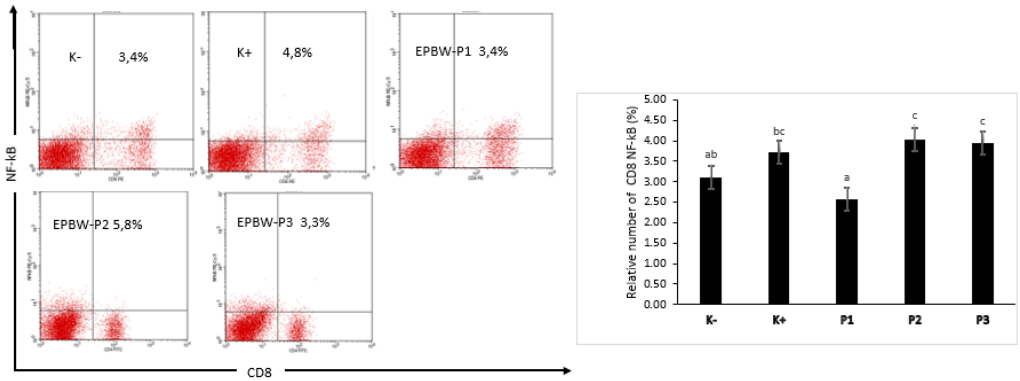


Figure 2. Graph of the Decreasing of NF-kB activation in CD8 cells in hyperglycemia mice. **Note:** K- = healthy control, K+ = pain control, P1 = hyperglycemia mice model with a treatment dose of 10 mg.kg^{-1} BW, P2 = hyperglycemia mice model with a treatment dose of 40 mg.kg^{-1} BW, P3 = hyperglycemia mice model with a treatment dose of 160 mg.kg^{-1} BW.

EPBW Decrease The Activation of NF-kB in CD8 Cells

The results of the analysis of flow cytometry and analysis of statistics of the number of NF-kB expressed from T lymphocyte CD8+ (Fig. 2). The positive control group, which is a hyperglycemia (K+) mouse model, the relative number T lymphocyte CD8+ cells that express NF-kB was the highest compared to the healthy control group (K-), in the amount of 3.1% ($p < 0.05$). The average healthy control was lower than the pain control at 3.7% but significantly different from the average relative number of cells expressing NF-kB in the hyperglycemia mice model P1 group 2.5%, but not different in P2 group by 4.04% and P3 3.9% are indicated by the notation in Figure 2.

EPBW DecreaseThe Blood Glucose Level

The results of measurement of blood sugar levels in hyperglycemia mice conducted every three days for 15 days of treatment showed a decrease. It is shown in Figure 3.

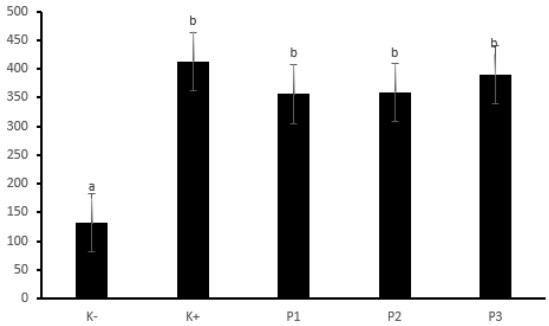


Figure 3. Graph of the decreasing fasting blood sugar levels. **Note:** K- = healthy control, K+ = pain control, P1 = hyperglycemia mice model with a treatment dose of 10 mg.kg^{-1} BW, P2 = hyperglycemia mice model with a treatment dose of 40 mg.kg^{-1} BW, P3 = hyperglycemia mice model with a treatment dose of 160 mg.kg^{-1} BW.

Negative control mice had blood sugar levels of 131 mg.dL^{-1}, whereas hyperglycemia model mice on positive controls had blood sugar levels of 413 mg.dL^{-1}. There was a decrease in all treatments with an average treatment of P1 356 mg.dL^{-1}, P2 of 359 mg.dL^{-1} and P3 389 mg.dL^{-1}.

Discussion

The combination of EPBW extracts as an antidiabetic to reduce NF-KB activation, proinflammatory factors, and reduce blood sugar levels in hyperglycemia mice models. Hyperglycemia is one of the first symptoms of diabetes [7]. Diabetogenic effects on streptozotocin administration will be initiated by reactive oxygen species (ROS) through direct toxic effects on GLUT 2, the cytokine action of TNF-α, and INF-γ due to stimulation of dependent T cells. In response to streptozotocin, Th-1 type cytokines and other immune cells produce ROS that activates NF-kB [8].

The use of the hyperglycemia mice model treated with EPBW extract on CD4 and CD8 cells showed a decrease in the number of activated NF-kB. As in diagrams and tables, NF-kB regulates the expression of most immunomodulatory factors, including cytokines, chemokines, adhesion molecules, antimicrobial factors, cell cycle regulators, and cell survival factors [9]. The decreased activation of NF-kB in the treatment group will affect the proliferation and activation of T and B cells. Also, it will affect the number of cytokines such as (IL-1, IL-6, TNF-α) that play a role in responding to the antigens or microorganisms and play a role in the inflammatory process [10].

Decreased amount of activated NF-kB will affect the number of cytokines produced during activation of NF-kB, a protein that can induce transcription of several types of genes. It can

ISSN. 2087-2852
E-ISSN. 2338-1655

stimulate inflammation by inducing the production of various inflammatory mediators, such as TNF-α, IFN-γ [11].

STZ induction in the Hyperglycemia mice model can trigger necrosis because it is toxic as in previous studies, that STZ could destroy pancreatic islet through necrosis mechanism. The damage of pancreatic beta cells is characterized by an increase in fasting blood sugar [12]. EPBW contains several active compounds that can reduce blood sugar levels. The decrease of blood glucose levels in the hyperglycemia mice model was caused by several active compounds found in *Momordica charantia*, e.g. sitosteryl glucoside and stigmasteryl glucoside. The combination of these compositions forms charantin, which is a potential substance with antidiabetic properties [5]. In addition, charantin can stimulate insulin secretion. It is due to increased recovery of pancreatic B cells because these substances can improve and inhibit pancreatic B cell death [13].

The function of Saponins in EPBW is as anti-hyperglycemia agents by preventing glucose uptake in the brush border in the small intestine. Flavonoids are also one of the active substances contained in EPBW, which is an alpha-glucosidase enzyme inhibitor that is used to delay the absorption of carbohydrates so that the blood glucose levels will decrease [5].

CONCLUSION

The combination of EPBW in hyperglycemia mice gave good results in reducing the amount of NF-kB activation. It supported by the decrease in blood sugar levels of hyperglycemia mice. A dose of 10 $mg.kg^{-1}$ BW is considered effective in reducing the amount of NF-kB activation as well as in reducing blood glucose levels in mice.

REFERENCES

[1] Minsitry of Health, Repuclic of Indonesia. 2018. Info datin pusat data dan informasi. Minsitry of Health. Jakarta.

[2] Almabrouk, S.K., N. Dwi, S. Rahayu, M. Rifa'i. 2019. The effect of *Moringa oleifera* leaves and VipAlbumin® on the immune system of diabetes mellitus balb/c mice model. *J. Exp. Life Sci.* 9(3). 170-175.

[3] Meadler, K., G.A. Spinas, P. Sergeev, M. Weber, A. Fontana, N. Kaiser, M.Y. Donath. 2001. Glucose induces beta-cell apoptosis via upregulation of the Fas receptor in human islets. *Diabetes.* 50(8). 1683-1690.

[4] Binti Hasim, N. 2014. Study on bioactive compound degradation from belimbing buluh (*Averrhoa Bilimbi*). Thesis. Faculty of Chemical and Natural Resources Engineering. Universiti Malaysia Pahang.

[5] Chanda, R., A. Samadder, J. Banerjee, C. Author. 2019. Anti-diabetic activity of *Momordica charantia* or bitter melon: a review. *Acta Scientific Pharmaceut. Sci.* 3(5). 2581-5423.

[6] Afifah, U.N. 2017. Uji aktivitas antidiabetes ekstrak etanol 96% Buah Pare (*Momordica charantia* L.) terhadap tikus jantan galur wistar yang diinduksi aloksan. Study Program of Pharmacy, Faculty of Pharmacy, Muhammadiyah University of Surakarta. 1-13.

[7] Rifa'i, M., N. Widodo. 2014. Significance of propolis administration for homeostasis of CD4+CD25+ T immunoregulatory cells controlling hyperglycemia. *SpringerPlus.* 3. 526.

[8] Lgssiar, A., M. Hassan, N. Friesen, F. Nicoletti, H. Gleichmann. 2004. Interleukin-11 inhibits NF-KB and AP-1 Activation in islets and prevents diabetes induced with streptozotocin in mice. *Exp. Biol. Med.* 15. 425-436.

[9] Oh, H., S. Ghosh. 2013. NF-κB: Roles and regulation in different CD4+ T-cell subsets. *Immunol. Rev.* 252(1). 41-51.

[10] Caamaño, J., C.A. Hunter. 2002. NF-κB family of transcription factors: Central regulators ofinnate and adaptive immune functions. *Clin. Microbiol. Rev.* 15(3). 414-429.

[11] Dwijayanti, D.R., M.S. Djati, M. Rifai 2015. Decreasing the expression level of macrophage cell, pro-inflammatory cytokines and NF-κb by using Vipalbumin® in vitro. *Asian J. Cell Biol.* 10(2). 43-56.

[12] Zamroni, A., E. Zubaidah, M. Rifa'i, S.B. Widjanarko. 2018. Anti-hyperglycemia and immunomodulatory activity of a polyherbal composed of *Sesbania grandiflora*, *Salacca zalacca* and *Acalypha indica*. *J. Exp. Life Sci.* 8(3). 184-192.

[13] Joseph, B., D. Jini. 2013. Antidiabetic effects of *Momordica charantia* (bitter melon) and its medicinal potency. *Asian Pac. J. Trop. Dis*. 3(2). 93-102

ISSN. 2087-2852
E-ISSN. 2338-1655

Physicochemical Characteristics and Consumer Acceptance of Bagged Indonesian Green Tea (*Camellia sinensis*) Formulated with Cinnamon Bark (*Cinnamomum burmannii*) and Lemon (*Citrus limon*) Peel

Adinda Kirana, Wenny Bekti Sunarharum*

Department of Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Brawijaya, Malang, Indonesia

Abstract

Green tea is a popular functional beverage mostly due to its high antioxidant activity. However, based on preliminary study, the taste such as its bitterness and flavour were somehow disliked by Indonesian consumers, and therefore formulation with other materials such as the addition of cinnamon and lemon peel may bring an advantage. This research was aimed to study the effect of formulation of bagged Indonesian green tea with cinnamon and lemon peel to the physicochemical characteristics of the tea and its consumer acceptance. Evaluation on the effect of different steeping time while brewing those tea formulas was also reported. The formula being investigated was F1, F2, and F3 with green tea: cinnamon: lemon peel at the ratio of 70:15:15, 70:12:18, and 70:9:21, respectively. Tea brewing was performed at 100°C, followed by a steeping time (L) at 1, 3, and 5 minutes. The results indicated that the addition of more cinnamon and longer steeping time had increased total phenol and antioxidant activity of the brewed tea. Based on the consumer acceptance test, the formula of green tea: cinnamon: lemon peel at 70:15:15 along with 3 minutes steeping time, was generally scored highest for degree of liking but the ratio of 70:12:18 was the most accepted or preferred for aroma. The best treatment was achieved on the ratio of green tea:cinnamon:lemon peel = 70:12:18, under 5 minutes steeping time. The best treatment provided a pH of 5.13, lightness (L*) at 44.93, redness(a*) at -2.50, yellowness (b*) at 16.93, total phenol of 59.82 mgGAE.g^{-1}, and IC_{50} of 59.39 ppm.

Keywords: Bagged Indonesian Green Tea, Cinnamon, Lemon Peel, Steeping Time

INTRODUCTION

The role of tea commodity in the world has been always withstand for its benefits for body requisite. Tea meets with our need for thirst, nutrition, and a particular taste. Indonesia is known as the fifth-largest tea producing country after China, India, Kenya, and Sri Lanka, typically for black, oolong, and green tea [1]. Green tea is one type of tea that can meet the body's nutritional needs because of its antioxidant compounds, such as polyphenols [2]. Green tea is superior comparing to other kinds of tea because green tea doesn't go through an enzymatic oxidation process. Therefore its antioxidant is more active and beneficial for the body [3]. But in addition to these advantages, green tea has a bitter taste that limits its consumption [4]. Our preliminary study also revealed that the bitterness and flavour of Indonesian green tea were somehow disliked by Indonesian consumers. Therefore formulation with other materials may bring benefits in terms of consumer's perspectives.

Out of several ingredients to be added into tea offered through an online survey, the majority of consumers (87.5%) mentioned preference to cinnamon and lemon peel. These two materials were locally and abundantly available in Indonesia. Cinnamon contains cinnamaldehyde, an essential compound that may contribute to sensory characteristics due to its ability to mask off-flavor. The compound is also useful because of its antioxidant properties, which are stronger than other herbs [5]. Different from cinnamon, lemon peels have a low antioxidant (793 ppm) [6]. However, its importance is mostly to alter the sensory character of the final product as previously reported in the study in dragon fruit peel tea and stevia, where 40% addition of lemon peel had improved its sensory characteristics in terms of taste, aroma, and overall flavour [7].

In the preparation of tea beverages, the dried tea leaves or tea in a bag (bagged tea) are generally brewed using hot water. The tea in contact with water was then allowed to soak for a few minutes to let the flavour and chemical components released in the extract, a processed called as steeping. The brewing process, especially the steeping time, has a significant role in the quality of the flavor and color of the brewed tea [8]. Generally, steeping time for tea was performed at 2-3 minutes [9]. Steeping for 5 minutes is proven to be able to extract 50% of tea polyphenols. However, longer steeping for

* Correspondence Address:
Wenny Bekti Sunarharum
Email : wbsunarharum@ub.ac.id
Address : Dept. Agricultural Product Technology, Universitas Brawijaya, Veteran Malang, 65145.

ISSN. 2087-2852
E-ISSN. 2338-1655

more than 10 minutes may cause tannin to detach and tea becomes even bitter [10].

Previous research on snake fruit peel herbal tea drinks with the addition of fragrant pandan leaves and cinnamon [11], had reported the importance of ingredients ratio in the formula to the characteristics and quality of the product. This research was aimed to study the effect of the formulation of bagged Indonesian green tea with cinnamon and lemon peel to the physicochemical characteristics of the tea and its consumer acceptance. The effect of different steeping times while brewing was also being investigated.

MATERIALS AND METHODS

Materials

The main materials used in this study were AA quality green tea (Wonosari Malang Tea Plantation), KM quality cinnamon bark (PT. Grencofe Premium Indonesia), and lemon peel (UKM Sari Lemon Gemeli, Batu). Those materials were dried using cabinet dryer and ground into powder (20 mesh).

Analytical materials included DPPH (SigmaAldrich), Folin-Ciocalteau (Merck), Na_2CO_3 (Merck), methanol pro analysis (Smartlab), ethanol pro analysis (Smartlab), gallic acid (Merck). Aquadest, aluminum foil, filter paper, paper teabag, and small paper cups were purchased from a local shop.

Preliminary Study

Stage one of the preliminary study was an online survey. The online method was used to collect data from 440 correspondents across Indonesia. The survey was aimed to get some insights on which kind of tea and additional ingredients for the tea formula that Indonesian consumers preferred, and thus giving the basis for product development. The participants of this survey were consumers mainly from the age of 17-25 years old (82.7%), men (71.4%), Javanese (65%), and university students (70.2%). The results were used for formulas that are further tested in this research.

Stage two was determining the percentage of materials in the formulation and the range of steeping time during the brewing process. The author follows the formula used by previous research on green tea with different additional ingredients [12], and modify it by using 50%, 60%, 70%, 80%, 90% of green tea. The trial was performed by employing limited consumer panellists (10 people) to test sensory acceptance. The range of steeping time during tea brewing was selected based on the Indonesian National Standard (SNI 01-4324-1996) and similar research about steeping time-related to polyphenol compounds [10,13]. The result of the preliminary study gave the formula and tea steeping time used in the research.

Research Design

The study was conducted using a factorial randomized block design with two factors. The first factor was the ratio of green tea: cinnamon:lemon peel with three formulations, i.e. F1 (70:15:15), F2 (70:12:18), and F3 (70:9:21), in percentage. The second factor was three levels of steeping time (L) at 1, 3, and 5 minutes. The analysis was conducted in triplicates. The best treatment was determined using the Multiple Attribute method by Zeleny [13].

Tea Preparation

Green tea powder, cinnamon powder, and lemon peel powder (20 mesh) were weighed according to the formula and bagged in a paper teabag ea. ± 2 g. This bagged tea was further brewed with hot water (100°C) before further steeping for certain times based on the designed treatments.

Physicochemical Analysis

All samples (dry tea formula and brewed tea) were analysed for color by measuring Lightness (L*), redness (a*), and yellowness (b*) by using color reader (CR-10) [14], pH by using pH meter (Senz pH) [15], and moisture content by using AOAC gravimetric method [16], total phenol [17] and antioxidant activity [18]. For total phenol analysis [17], the sample (0.5 mL) was added to a 10 mL Folin Ciocalteau 10% solution, then incubated for 5 minutes at room temperature (±28°C). Then, 2 mL of Na_2CO_3 7.5% added before further homogenization and incubation for 30 minutes in the dark. The absorbance was measured at a maximum wavelength of 750 nm. Total phenol levels were expressed as gallic acid equivalent ($mgGAE.g^{-1}$).

The antioxidant activity was measured by IC_{50} analysis [18], where 2 mL samples were reacted with 2 mL DPPH 0.2 mM and homogenized. The mixtures was further incubated at room temperature (±28°C) in the dark for 30 minutes. Absorbance were measured using a spectrophotometer (Thermo Scientific Genesys 110s UV-Vis) at a maximum wavelength of 517 nm.

Consumer Acceptance Test

Consumer acceptance of the brewed tea was evaluated by using the hedonic method [19]. For

ISSN. 2087-2852
E-ISSN. 2338-1655

this purpose, as many as 140 consumer panellists were employed to evaluate 9 samples for the degree of liking. Panellists were asked to rate six parameters i.e. color, aroma, taste, aftertaste, appearance, and overall liking from 1 to 9.

Statistical Data Analysis

Data was collected and tabulated in Microsoft Excel 2013 software (Microsoft Corp., Redmond, Washington, USA). Analysis of Variance (ANOVA) and further post-hoc analysis was performed using Minitab 17 statistical software (Minitab Inc., State College, Pennsylvania, USA).

RESULTS AND DISCUSSION

Evaluation on Raw Materials

Based on Table 1, it can be seen that each material has different chemical and physical characteristics. It should be noted that the differences are not only solely due to the different material but could also be the origin of raw materials, storage conditions, humidity of the surrounding air, post-harvest processing, particle size, harvesting process, the duration of the process before being packed, the type of machine/tool during the process, the method and the purpose of extraction, type of quality, method of drying and varieties of material [20].

The different characteristics are due to the compounds contained in the material. Lemon peels contain organic acids, such as citric acid, malic acid, oxalic acid, and malonic acid [21]. Cinnamon contains cinnamic acid, cinnamid, vanillic acid, gallic acid, protocatechuic acid, caffeic acid, ferulic acid, p-coumaric [22]. Green tea contains several organic acids, including ascorbic acid, malic acid, oxalate, gallate, chlorogenic, and succinate [23].

Table 1. Characteristics of teabag raw materials

Parameter	Green Tea	Cinnamon	Lemon Peel
water content (%)	7.82 ± 0.34	8.88 ± 0.55	9.67 ± 0.35
L*	47.30 ± 0.95	53.73 ± 0.83	70.77 ± 2.80
a*	-2.27 ± 0.15	8.97 ± 0.51	1.60 ± 0.10
b*	11.57 ± 0.74	20.73 ± 0.59	30.67 ± 0.21
pH	5.23 ± 0.15	5.17 ± 0.06	4.43 ± 0.12
Total phenol ($mgGAE.g^{-1}$)	257.37 ± 0.55	81.93 ± 0.29	30.87 ± 0.24
Antioxidant activity IC_{50} (ppm)	16.37 ± 0.21	54.54 ± 0.48	294.18 ± 4.55

Note: Data mean ± standard deviation (n=3)

Formulation of Materials

Based on Table 2, it can be seen that the higher the amount of lemon peel would increase the water content. In a less optimal drying process, water will still be available in the albedo part, therefore the water content is still quite high. Albedo's function is to supply nutrients and water from trees [24]. It is also known that lightness (L*), and yellowness (b*) are increased while decreasing redness (a*). The increase in L* and b* value are thought due to the degradation of chlorophyll pigments into carotenoids and β-cryptoxanthin during fruit ripening resulting in yellowish color and lightness [25]. The a* value decreased with the reduction of cinnamon suggested due to the loss of darkness contribution such as from cinnamaldehyde and anthocyanin [11].

Table 2. Characteristics of dry teabag formulations

Parameter	Treatment Ratio of Green Tea:Cinnamon: Lemon Peel (%)		
	F1 (70:15:15)	F2 (70:12:18)	F3(70:9:21)
Water content (%)	8.80 ± 0.79	9.34 ± 0.46	9.99 ± 0.29
L*	46.83 ± 1.04^{c}	49.17 ± 0.81^{b}	52.33 ± 0.57^{a}
a*	0.48 ± 0.03^{a}	0.38 ± 0.03^{b}	-0.20 ± 0.00^{c}
b*	14.63 ± 0.21^{b}	15.70 ± 0.53^{ab}	16.90 ± 0.96^{a}
pH	4.93 ± 0.06	4.87 ± 0.05	4.80 ± 0.10
Total phenol ($mgGAE.g^{-1}$)	236.10 ± 0.95^{a}	225.46 ± 0.73^{b}	192.76 ± 0.82^{c}
Antioxidant activity IC_{50} (ppm)	16.46 ± 0.30^{c}	17.61 ± 0.10^{b}	19.01 ± 0.44^{a}

Note: Data mean ± standard deviation (n=3). Numbers accompanied by different notations showed significant differences (α = 0.05)

Cinnamon contains cinnamic acid, gallic acid [22], cinnamaldehyde, eugenol >80%, and others [26]. These acidic compounds can play a role in the pH of cinnamon and affect formulation pH. Those acidic compounds also have a role with polyphenols such as epigallocatechin gallate (EGCg) in green tea, terpineol in cinnamon [27], and D-limonene in lemon peel [28]. Table 2 indicates the decrease in total phenol and antioxidant activity when more lemon peel added. It is suggested due to the lower amount of total phenols and antioxidant activity in the lemon peel as compared to that of cinnamon. Previous research had also reported that lemon peel has a week antioxidant level [29].

Evaluation on the Brewed Tea

The brewed tea was analysed for color, pH, total phenol, IC_{50} values, and the level of panelist preference.

1. Color Analysis

Results showed that the formulation of green tea, cinnamon and lemon peel, steeping time, and interactions between two factors has a significant effect (α = 0.05) on lightness (L*), redness (a*) but not on yellowness (b*) of brewed tea. Table 3 showed that higher lemon peel proportion would increase L* and b* value

ISSN. 2087-2852
E-ISSN. 2338-1655

of brewed tea while decreasing a* value. The L* value increase with the reduction in the proportion of cinnamon due to the loss of red color and lightness contribution from cinnamon. The results also showed that brewed tea has more b* than a* value, suggested due to more carotene compound that contributes to the yellow color while chlorophyll, carotene, pheophytin, xanthophyll, and pheophorbide in green tea and cinnamon might contribute to a* value [30].

This study had also indicated that the longer steeping time increased the L*, a* and b* value, suggested due to more compounds extracted and interacted with others. The high temperature might result in color change due to chlorophyll degradation into pheophytin, which looks grayish-green [31].

2. pH Measurement

The results of pH measurement of the samples are provided in Table 3. It can be seen that proportion of green tea, cinnamon, and lemon peel, steeping time had a significant effect on the pH of brewed tea (α = 0.05), but the interaction between two factors was not significant (α = 0.05). The higher proportion of lemon peel in a teabag and longer steeping time was found to decrease the pH of brewed tea. It is suggested due to the contribution of some components in teabag materials, such as organic acid (citric acid) in lemon peel [21], and the more acids dissolved [32].

3. Total Phenol

Table 3 showed the result of total phenol analysis. The ANOVA indicated that the proportion of green tea, cinnamon, and lemon peel, steeping time, and interaction between the two factors had a significant effect (α = 0.05) on total phenols of brewed tea. It revealed that the higher proportion of cinnamon tends to increase the total phenolic content of brewed tea (Table 3) since cinnamon has higher phenol than lemon peel (Table 1). One of important phenolic compounds in cinnamon is eugenol [27]. The increase in steeping time is found to increase total phenol. Again, this could be explained due to more extraction of phenolic compounds during steeping until it reaches the optimum point, where no more compounds can be extracted.

4. Antioxidant Activity

Antioxidant activity of the brewed tea (Table 3) expressed as IC_{50} was found to be significantly different (α = 0.05) as influenced by different tea formulation and steeping time. The IC_{50} ranged approximately from 47.59 up to 115.49 ppm. Table 3 showed that a higher proportion of cinnamon tends to increase the antioxidant activity of brewed tea (or the decrease in IC_{50} value). It is suggested due to the incorporation of several phenolic compounds, which are an active antioxidant from cinnamon such as cinnamaldehyde, cinnamyl acetate, and benzyl benzoate to the extract. The longer the steeping time was also found to decrease the IC_{50} of the corresponding brew, meaning that the antioxidant activity level was higher. The three formula (F1, F2, F3) showed a similar trend where the 5 minutes extraction time could increase antioxidant activity into two-fold, as shown by a decrease in IC_{50} up to almost half as compared to the 1-minute extraction or steeping time. Longer time allows more phenols and antioxidant compounds to be extracted into the brewed/infused tea.

Table 3. Characteristics of the brewed tea

Treatment	Parameter					
	L*	a*	b*	pH	Total phenol (mgGAE.g^{-1})	IC_{50} (ppm)
F1L1	54.00 ± 1.93^{ab}	-3.30 ± 0.20^{c}	8.30 ± 0.82	5.33 ± 0.06	38.36 ± 2.90^{e}	81.87 ± 4.67^{bc}
F1L2	41.63 ± 1.263^{e}	-2.63 ± 0.31^{b}	13.07 ± 0.57	5.23 ± 0.06	60.97 ± 0.69^{ab}	68.07 ± 1.57^{d}
F1L3	35.27 ± 3.37^{f}	-0.57 ± 0.06^{a}	15.40 ± 0.89	5.17 ± 0.12	62.43 ± 0.15^{a}	47.59 ± 1.30^{f}
F2L1	54.33 ± 3.26^{ab}	-3.90 ± 0.30^{d}	9.03 ±0.57	5.30 ± 0.00	36.20 ± 0.48^{f}	85.64 ± 1.70^{b}
F2L2	48.00 ± 0.36^{cd}	-2.90 ± 0.27^{bc}	16.87 ± 1.20	5.17 ± 0.06	55.98 ± 0.42^{c}	79.89 ± 0.48^{c}
F2L3	44.93 ± 3.87^{de}	-2.50 ± 0.27^{b}	16.93 ± 1.60	5.13 ± 0.06	59.82 ± 0.20^{b}	59.39 ± 1.67^{e}
F3L1	55.57 ± 2.58^{a}	-6.03 ± 0.64^{e}	10.43 ± 0.71	5.27 ± 0.06	35.41 ± 0.67^{f}	115.49 ± 4.38^{a}
F3L2	50.90 ± 2.52^{bc}	-3.17 ± 0.21^{c}	17.23 ± 1.70	5.13 ± 0.06	39.38 ± 0.38^{e}	81.27 ± 2.94^{bc}
F3L3	48.73 ± 1.03^{cd}	-2.53 ± 0.31^{b}	17.47 ± 0.97	5.00 ± 0.10	47.63 ± 0.77^{d}	66.56 ± 2.32^{d}

Note: F1= green tea: cinnamon: lemon peel (70:15:15), F2 (70:12:18) and F3 (70:9:21), L1= steeping time at 1 minute, L2 = 3 minutes, L3 = 5 minutes, Data mean ± standard deviation (n=3). Numbers accompanied by different notations showed significant differences (α = 0.05)

ISSN. 2087-2852
E-ISSN. 2338-1655

5. Consumer Acceptance

In the sensory evaluation, there were six parameters (color, aroma, taste, appearance, aftertaste, and overall liking) scored by 140 consumer panelists. Score from 1 to 9 was used in the test to know the degree of liking or consumer acceptance. The result of this test can be seen in Table 4, where the formula of green tea: cinnamon: lemon peel at 70:15:15 along with 3 minutes steeping time (F1L2) was scored highest for all parameters except for aroma. The consumers preferred green tea: cinnamon: lemon peel at 70:12:18 combined with 5 minutes steeping time (F2L3) for aroma. It is more likely that consumer's preference was driven by the aroma of the brew, where lemony aroma can offer a balance with a strong cinnamon aroma. The more lemon peel adds more acidity and lemon notes responsible for creating balance and reduce the dominance of cinnamon aroma. Moreover, the longer steeping time of 5 minutes was suggested to increase the extraction of aromatic compounds, essential oils, or flavour compounds from cinnamon and lemon peel into the brew.

Table 4. Panelists' preference level for the brewed tea

Treatment	Parameter					
	color	aroma	taste	after-taste	appearance	overall liking
F1L1	5.83	5.54	5.64	5.72	5.97	5.87
F1L2	**6.21**	5.79	**6.32**	**6.14**	**6.12**	**6.99**
F1L3	5.91	5.89	5.34	5.25	6.06	5.82
F2L1	6.03	5.62	5.60	5.64	6.01	5.90
F2L2	6.08	5.82	5.53	5.44	5.84	5.91
F2L3	5.83	**5.95**	5.59	5.46	5.72	5.84
F3L1	5.50	5.41	5.56	5.66	5.43	5.71
F3L2	5.98	5.59	5.29	5.48	6.03	5.72
F3L3	5.97	5.94	5.59	5.40	5.96	5.80

Note: F1= green tea: cinnamon: lemon peel (70:15:15), F2 (70:12:18) and F3 (70:9:21), L1= steeping time at 1 minute, L2 = 3 minutes, L3 = 5 minutes. Bold numbers indicate the highest value of liking (most preferred)

6. Best Formula and Steeping Time Selection

The best treatment in this research (formula and steeping time) is determined by the Multiple Criteria Decision-Making method [14], where all attributes are equally important. The involvement of all these attributes caused the ranking of each treatment to be different so that a different effectiveness index was also obtained. The determination of the best treatment can be calculated from all parameters measured i.e. physical, chemical, and organoleptic parameters with the lowest density distance.

The best treatment with the lowest value of the effectiveness index 0.56 was the F2L3, which is a formula of green tea: cinnamon: lemon peel of 70:12:18, combined with 5 minutes steeping time. The physical and chemical quality characteristics of the best tea formula is provided in Table 5.

Table 5. Characteristics of the best treatment(brewed tea)

Parameter	Best Treatment (F2L3)	Green Tea
pH	5.13	-
Color (L*a*b* value)	44.93* -2.50* 16.93*	-
Total phenol (mgGAE.g^{-1})	59.82	68.55
Antioxidant activity (IC_{50}) (ppm)	59.39	46.03

The chosen formula and steeping time has lower total phenol and antioxidant activity than pure green tea (Table 5), but the organoleptic characteristics were suggested to be better due to the incorporation of cinnamon and lemon peel. The addition of these two ingredients had improved flavour, in this case, cinnamon and lemon, giving sweetness, warm and lemony characteristics of the tea. The balance of these characters had contributed to an increase in consumer acceptance.

CONCLUSION

The incorporation of cinnamon and lemon peel in different proportion, as well as steeping time, had a significant influence on physical, chemical and organoleptic properties of brewed green tea to some extent, such as the effect on lightness (L*), redness (a*), yellowness (b*), pH, total phenol, and antioxidant activity value of IC_{50}. The interaction between the two factors or treatments is also affecting some of the parameters. The changes in brewed tea properties are suggested due to different compositions and interactions between components extracted during steeping. The formula of green tea: cinnamon: lemon peel at 70:15:15 along with 3 minutes steeping time was the most preferred by consumers, but it was not chosen as the best formula or treatment. The best treatment was the ratio of green tea:cinnamon:lemon peel = 70:12:18, under 5 minutes steeping time, that was chosen by considering not only consumer degree of liking but also other physicochemical parameters. The result had indicated the potential of the current formula as a functional product. However, further research will be required to justify the benefits and functionality of the product.

REFERENCES

[1] Chang, K. 2015. World tea production and trade: current and future development.

ISSN. 2087-2852
E-ISSN. 2338-1655

Food and Agriculture Organization of The United Nations. Rome.

[2] Balittri, J.T. 2013. Kandungan senyawa kimia pada daun teh. *Warta Penelitian Dan Pengembangan Tanaman Industri*. 19(3). 12-16.

[3] Sinija, V.R., H.N. Mishra. 2008. Green tea: health benefits. *J. Nutr. Environ. Med.* 17(4). 232-242.

[4] Kusanto, Y.S. 2013. Pengaruh variasi konsentrasi air seduhan teh hijau terhadap sifat fisikokimia dan organoleptik permen jelly teh hijau (*Camellia sinensis*). PhD Thesis. Faculty of Agricultural Technology, Universitas Katolik Widya Mandala Surabaya.

[5] Ravindran, P.N., K.N. Babu, M. Shylaja. 2004. Cinnamon and cassia the genus *Cinnamomum*: medicinal and aromatic plants–industrial profiles. CRC Press. Washington DC.

[6] Asendy, D.A., I.W.R. Widarta, K.A. Nocianitri. 2018. Pengaruh waktu maserasi terhadap aktivitas antioksidan ekstrak kulit buah jeruk lemon (*Citrus limon Linn*). *Jurnal Ilmu dan Teknologi Pangan*. 7. 102-109.

[7] Shofiati, A., M.A.M. Andriani, C. Anam. 2014. Kajian kapasitas antioksidan dan penerimaan sensoris teh celup kulit buah naga (pitaya fruit) dengan penambahan kulit jeruk lemon dan stevia. *Jurnal Teknosains Pangan.* 3(2). 5-13.

[8] Ramlah, R. 2017. Penentuan suhu dan waktu optimum penyeduhan daun teh hijau (*Camellia sinensis* L) P+ 2 terhadap kandungan antioksidan kafein, tannin dan katekin. PhD Thesis. Universitas Islam Negeri Alauddin Makassar.

[9] McAlpine, M.D., W.E. Ward. 2016. Influence of steep time on polyphenol content and antioxidant capacity of black, green, rooibos, and herbal teas. Beverages. 2(3). 17.

[10] Cleverdon, R., Y. Elhalaby, M.D. McAlpine, W. Gittings, W.E. Ward. 2018. Total polyphenol content and antioxidant capacity of tea bags: Comparison of black, green, red Rooibos, chamomile and peppermint over different steep times. Beverages. 4(1). 15.

[11] Anjani, P.P., S. Andrianty, T.D. Widyaningsih. 2015. Pengaruh penambahan pandan wangi dan kayu manis pada teh herbal kulit salak bagi penderita diabetes. *Jurnal Pangan dan Agroindustri*. 3(1). 203-214.

[12] Prasetyo, E. 2013. Formula bubuk "Teajes" instan pada filtrat teh hijau (*Camellia sinensis*) dan filtrat jahe (*Zingiber officinale*) dengan penambahan filtrat kayu secang (*Caesalpinia sappan*). PhD Thesis. Universitas Brawijaya. Malang.

[13] Zeleny, M. 1982. Multiple criteria decision making. McGraw-Hill. New York City.

[14] Juncai, H., H. Yaohua, H. Lixia, G. Kangquan, T. Satake. 2015. Classification of ripening stages of bananas based on support vector machine. *Int. J. Agr. Biol. Eng.* 8(6). 99-103.

[15] Angelia, I.O. 2017. Kandungan pH, total asam tertitrasi, padatan terlarut dan vitamin c pada beberapa komoditas hortikultura (pH content, total acidified acid, dissolved solids and vitamin c in some horticultural commodities). *J. Agritech Sci.* (JASc). 1(2). 68-74.

[16] AOAC. 1997. Official methods of analysis: association of official analytical chemist. Association of Official Analytical Chemists Inc. Arlington.

[17] Sharma, G.N., S.K. Dubey, N. Sati, J. Sanadya. 2011. Phytochemical screening and estimation of total phenolic content in Aegle marmelos seeds. *Int. J. Pharm. Clin. Res.* 2(3). 27-29.

[18] Molyneux, P. 2004. The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. *Songklanakarin J. Sci. Technol.* 26(2). 211-219.

[19] SSP. 2020. The 9-Point Hedonic Scale. Available at: https://www.sensorysociety.org/.

[20] Nwoseu, F., A. Kareem. 2015. Production of citric acid from lemon and cassava waste peels. *Nigerian J. Mater. Sci. Eng.* 6(1). 1-7.

[21] Clements, R.L. 1964. Organic acids in citrus fruits. I. Varietal differences a. *J. Food Sci.* 29(3). 276-280.

[22] Emilda, E. 2018. Efek senyawa bioaktif kayu manis *cinnamomum burmanii* nees ex. Bl.) Terhadap diabetes melitus: kajian pustaka. *Jurnal Fitofarmaka Indonesia*. 5(1). 246-252.

[23] Chatuverdula, V.S.P., R.I.S. Miguel, I. Prakash. 2012. Chemical constituents from polar fraction of Rubus suavissimus. *Organic Chem Current Res.* 1(1). DOI: 10.4172/2161-0401.1000101.

[24] Ekaputri, F. 2018. Pengaruh perbandingan kulit dan sari lemon dan konsentrasi kayu manis terhadap karakteristik selai lemon

(*Citrus limon burm f*.) secara organoleptik. Master Thesis. Universitas Pasundan.

[25] Conesa, A., F.C. Manera, J.M. Brotons, J.C. Fernandez-Zapata, I. Simón, S. Simón-Grao, M. Alfosea-Simón, J.M., Nicolás, J.M. Valverde, F. García-Sanchez. 2019. Changes in the content of chlorophylls and carotenoids in the rind of Fino 49 lemons during maturation and their relationship with parameters from the CIELAB color space. *Scientia Horticulturae*. 243. 252-260.

[26] Nabavi, S.F., A. Di Lorenzo, M. Izadi, E. Sobarzo-Sánchez, M. Daglia, S.M. Nabavi, 2015. Antibacterial effects of cinnamon: from farm to food, cosmetic and pharmaceutical industries. *Nutrients*. 7(9). 7729-7748.

[27] Praseptiangga. D., S.E. Invicta, L.U. Khasanah. 2019. Sensory and physicochemical characteristics of dark chocolate bar with addition of cinnamon (*Cinnamomum burmannii*) bark oleoresin microcapsule. J. Food Sci. Technol. 56(9). 4323-4332.

[28] Moosavy, M., P. Hassanzadeh, E. Mohammadzadeh, R. Mahmoudi, S. Khatibi, K. Mardani. 2017. Antioxidant and antimicrobial activities of essential oil of lemon (*Citrus limon*) peel in vitro and in a food model. *J. Food Qual. Hazards Control*, 4(2). 42-48.

[29] Juanda, D. 2015. Penetapan kadar total fenol dan aktivitas antioksidan dari jus buah lima spesies jeruk (*Citrus* sp.). *Jurnal farmasi Galenika*. 2(1). 36-42.

[30] Andarini, F., S. Yasni. 2018. Pengembangan minuman fungsional dari ekstrak kulit mundar. Jurnal Teknologi dan Industri Pangan. 29(1). 49-57.

[31] Kusmita, L., I. Puspitanningrum, L. Limantara. 2015. Identification, isolation and antioxidant activity of pheophytin from green tea (*Camellia sinensis* (L.) *Kuntze*). Procedia Chem. 14(14). 232-238.

[32] Ibrahim, A.M., Y. Yunianta, F.H. Sriherfyna. 2014. Pengaruh suhu dan lama waktu ekstraksi terhadap sifat kimia dan fisik pada pembuatan minuman sari jahe merah (*Zingiber officinale* var. Rubrum) dengan kombinasi penambahan madu sebagai pemanis. *Jurnal Pangan dan Agroindustri*. 3(2). 530-541.

MANUSCRIPT SUBMISSION

FOCUS AND SCOPE

Journal of Experimental Life Science (JELS) is scientific journal published by Graduate Program of Brawijaya University as distribution media of Indonesian researcher's results in life science to wider community. JELS is published in every four months. JELS published scientific papers in review, short report, and life sciences especially nanobiology, molecular biology and cellular biology. JELS is scientific journal that published compatible qualified articles to academic standard, scientific and all articles reviewed by expert in their field.

Journal of Experimental Life Science (JELS) have vision to become qualified reference media to publish the best and original research results, and become the foundation of science development through invention and innovation on cellular, molecular, and nanobiology rapidly to community.

Journal of Experimental Life Science (JELS) have objectives to published qualified articles on research's results of Indonesian researchers in life science scope. JELS encompasses articles which discuss basic principles on nature phenomenon with cellular, molecular, and nanobiology approach.

PEER REVIEW PROCESS

Publication of articles by JITODE is dependent primarily on their validity and coherence, as judged by peer reviewers, who are also asked whether the writing is comprehensible and how interesting they consider the article to be. All submitted manuscripts are read by the editorial staff and only those articles that seem most likely to meet our editorial criteria are sent for formal review. All forms of published correction may also be peer-reviewed at the discretion of the editors. Reviewer selection is critical to the publication process, and we base our choice on many factors, including expertise, reputation, and specific recommendations. The editors then make a decision based on the reviewers' advice, from among several possibilities:

Accepted, with or without editorial revisions
Invite the authors to revise their manuscript to address specific concerns before a final decision

Rejected, but indicate to the authors that further work might justify a resubmission

Rejected outright, typically on grounds of specialist interest, lack of novelty, insufficient conceptual advance or major technical and/or interpretational problems

PUBLICATION FREQUENCY

JELS publish 2 Issues per year until 2017. JELS started to publish 3 Issues per year since 2018.

OPEN ACCESS POLICY

This journal provides immediate open access to its content on the principle that making research freely available to the public supports a greater global exchange of knowledge.

COPYRIGHT NOTICE

Authors who publish with this journal agree to the following terms:

Authors retain copyright and grant the journal right of first publication with the work simultaneously licensed under a Creative Commons Attribution License that allows others to share the work with an acknowledgement of the work's authorship and initial publication in this journal.

Authors are able to enter into separate, additional contractual arrangements for the non-exclusive distribution of the journal's published version of the work (e.g., post it to an institutional repository or publish it in a book), with an acknowledgement of its initial publication in this journal.

Authors are permitted and encouraged to post their work online (e.g., in institutional repositories or on their website) prior to and during the submission process, as it can lead to productive exchanges, as well as earlier and greater citation of published work (The Effect of Open Access).

PRIVACY STATEMENT

The names and email addresses entered in this journal site will be used exclusively for the stated purposes of this journal and will not be made available for any other purpose or to any other party.

ETHICS PUBLICATION

Research that using animal, human, and clinical testing is should already have ethical clearance certificate from authorized institution.

Title Typed in Bold, Capitalize each First Letter of Each Word, Except Conjunctive, *Scientific name* should not be Abbreviated (Calibri 14 Bold Center, should not exceed 12 words, except conjuctive)

First Author[1]*, Second Author[2], Third Author[3] (Calibri 12 Center, without title)

[1]First Author Affiliation, Correspondence author should be indicated by * symbol (Calibri 9 Center)
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia
[3]Laboratorium of Physiology, Faculty of Medicine, University of Brawijaya, Malang, Indonesia

Abstract (Calibri 9 Bold Center)

This article illustrates preparation of your paper using MS-WORD (.doc or .rtf). Manuscript was numbered consecutively. Main text typed in two columns (67 characters), except title and abstract in one column. The manuscript should be written in English. The length of manuscript should not exceed 10 pages including table and figure in this format using A4 paper single space. The text should be in the margin of 3 cm up, down and left side, 2.5 cm on right side. Abstract includes the research purposes, research method and research results in one paragraph of *essay,* not *enumerative*. No citation in abstract. Abstract should not exceed 200 words. Keywords typed after abstract. (Calibri 9 Justify).

Keywords: manuscript, English, format, 5 words maximum (Calibri 9 Left)

INTRODUCTION* (Calibri 10 Bold, Left, Capslock)

All submitted manuscripts should contain original research which not previously published and not under consideration for publication elsewhere. Articles must be written in ENGLISH and manuscripts may be submitted for consideration as research report articles, short reports or reviews.

The introduction explains the background of the problem, the study of literature and research purposes. Some initial introduction paragraphs explain the problem and background to these problems [1]. The next few paragraphs explain the study of literature that contains recent knowledge development which is directly related to the issues. The last paragraph of the introductory section contains a description of the purposes of the study. (Calibri 10 Justify)

MATERIAL AND METHOD (Calibri 10 Bold, Left, Capslock)

This section describes the types of methods (qualitative, quantitative or mixed-method) with details of methods of data collection and data analysis [2]. This section also describes the perspective that underlying the selection of a particular method. (Calibri 10 Justify)

Correspondence address: (Calibri 8 Bold, Left)
Full name of correspondence author
Email : sapto@jurnal.ub.ac.id
Address : afiliation address include post code

Data Collection (Calibri 10 Bold, Left)

Explain the data collection methods, i.e. surveys, observations or archive, accompanied by details of the use of such methods. This section also describes the population, sampling and sample selection methods. (Calibri 10 Justify)

The use of English language should followed proper grammar and terms. Name of organism shoul be followed by its full scientific name in the first mention, in *italic* [3]. Author of the scientific name and the word of "var." typed regular. Example: *Stellaria saxatillis* Buch. Ham. First abbreviation typed in colon after the abbreviated phrase.

Author must use International Standard Unit (SI). Negative exponent used to show the denominator unit. Example: g l^{-1}, instead of g/l. The unit spaced after the numbers, except percentage [4]. Example: 25 g l^{-1}, instead of 25gl^{-1}; 35% instead of 35 %. Decimal typed in dot (not coma). All tables and figures should be mentioned in the text.

RESULT AND DISCUSSION (Calibri 10 Bold, Left, Capslock)

This section contains the results of the analysis and interpretation or discussion of the results of the analysis. Describe a structured, detailed, complete and concise explanation, so that the reader can follow the flow of analysis and thinking of researchers [5]. Part of the results study should be integrated with the results of the

analysis and the results and discussion are not separated.

Table

Table should be submitted within the manuscript and in separated file of *Microsoft Excel* (xls.). Table whould not exceed 8 cm (one column) and 17 cm (two columns). Table should be embedded in different page after references.

Table should be numbered in sequence. Table title should be brief and clear above the table, with uppercase in initial sentence. Vertical line should not be used. Footnote use number with colon and superscripted. Symbol of (*) or (**) was used to show difference in confidence interval of 95 and 99%.

Table 1. Example of the Table (Calibri 8.5 Left)

No	Point (Calibri 8.5 Justify)	Description
1		
2		
3		
4		
5		

Sources: Journal of PPSUB (Calibri 8.5 Left)

Figures

Figures should be in high resolution and well contrast in JPEG or PDF with the following conditions:

- Monochrome image (line art), figures of black and white diagram (solid/no shades of gray), resolution 1000-1200 dpi (dot per inch).
- Combination Halftone, combine figure and text (image containing text) and coloured graphic or in grayscale format. Resolution 600-900 dpi.
- Halftone, coloured figure or grayscale format without text. Resolution 300 dpi.
- Black and white figure should be in the grayscale mode, while coloured figures should be in RGB mode.
- Figure should not exceed the width of 8 cm (one column), 12.5 cm (1.5 columns) or 17 cm (two columns).
- Figures title typed clearly below the figure.
- Figure with pointing arrow should be grouped (grouping).
- Figures were recommended in black and white.
- Legend or figure description should be clear and complete. If compressed, the figure should be readable.
- Statistic graphic should be supplemented with data sources.
- If the figures come from the third party, it should have the copyright transfer from the sources.

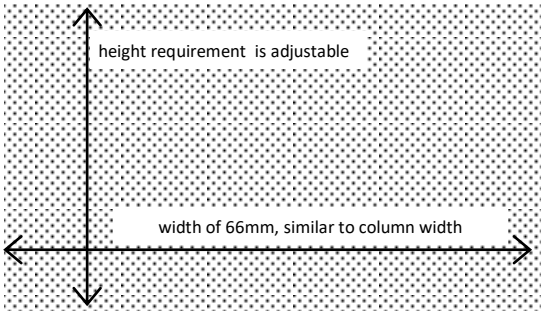


Figure 1. Illustration of Dimensional Figure of one column width. Figure dimension adjusted to the width of one column. Name the figure (diagram) written below the image. (Calibri 8.5 Justify)

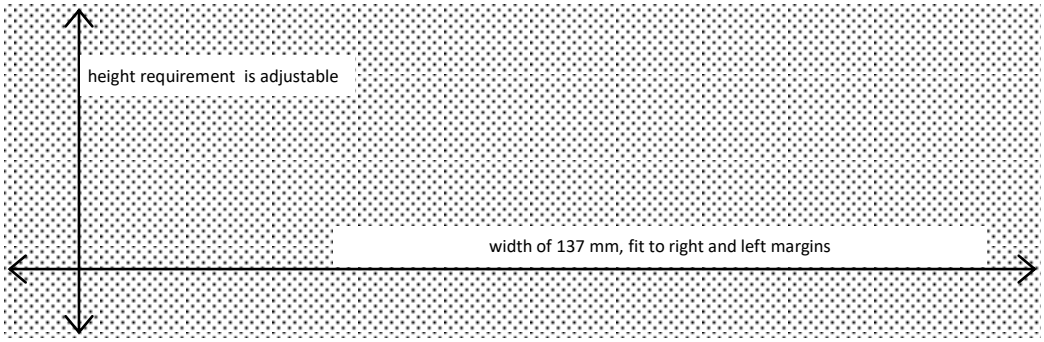


Figure 2. . Illustration of Dimensional Figure of two column width. Figure dimension adjusted to the width of two columns (137 mm). Figure were align top or bottom of the page. (Calibri 8.5 Justify)

References

1. Primary references include journal, patent, dissertation, thesis, paper in proceeding and text book.
2. Avoid self citation.
3. Author should avoid reference in reference, popular book, and internet reference except journal and private ana state institution.
4. Author was not allowed to use abstract as references.
5. References should been published (book, research journal or proceeding). Unpublished references or not displayed data can not be used as references.
6. References typed in numbering list (format number 1,2,3,…), ordered sequentially as they appear in the text (system of Vancouver or author-number style).
7. Citation in the manuscript typed only the references number (not the author and year), example: Obesity is an accumulation of fat in large quantities which would cause excessive body weight (overweight) [1]. Obesity is a risk factor of diabetic, hypertension dan atherosclerosis [2].

CONCLUSION (Calibri 10 Bold, Left, Capslock)

Conclusion of the study's findings are written in brief, concise and solid, without more additional new interpretation. This section can also be written on research novelty, advantages and disadvantages of the research, as well as recommendations for future research.(Calibri 10 Justify)

ACKNOWLEDGEMENT (Calibri 10 Bold, Left, Capslock)

This section describes gratitude to those who have helped in substance as well as financially. (Calibri 10 Justify)

REFERENCES (Calibri 10 Bold, Left, Capslock)

[1]. (Calibri 10 Justify, citation labelling by references numbering)
[2]. Vander, A., J. Sherman., D. Luciano. 2001. Human Physiology: The Mecanisms of Body Function. McGraw-Hill Higher Education. New York. (Book)
[3]. Shi, Z., M. Rifa'i, Y. Lee, K. Isobe, H. Suzuki. 2007. Importance of CD80/CD86-CD28 interaction in the recognation of target cells by $CD8^{+}CD122^{+}$ regulatory T cells. *Journal Immunology.* 124. 1:121-128. (Article in Journal)
[4]. Syafi'i, M., Hakim, L., dan Yanuwiyadi, B. 2010. Potential Analysis of Indigenous Knowledge (IK) in Ngadas Village as Tourism Attraction. pp. 217-234. In: Widodo, Y. Noviantari (eds.) Proceed-ing *Basic Science National Seminar 7* Vol.4. Universitas Brawijaya, Malang. (Article within conference proceeding)
[5]. Dean, R.G. 1990. Freak waves: A possible explanation. p. 1-65. *In* Torum, A., O.T. Gudmestad (eds). Water wave kinetics. CRC Press. New York. (Chapter in a Book)
[6]. Astuti, A.M. 2008. The Effect of Water Fraction of *Stellaria* sp. on the Content of TNF-α in Mice (*Mus musculus* BALB-C). Thesis. Department of Biology. University of Brawijaya. Malang. (Thesis)

Cover Images:

3D Structure of EGCG (*Epigallocatechin-3-Gallate*)
Green Tea Component

Created by:
Prof. Widodo, S.Si.,M.Si.,Ph.D MED Sc.

Address:

Building B, 1st Floor, Graduate School, University of Brawijaya
Jl. Mayor Jenderal Haryono 169, Malang, 65145
Telp: (0341) 571260 ; Fax: (0341) 580801
Email: jels@ub.ac.id
Web: jels.ub.ac.id

9 772087 285007