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Original Research

Second allogeneic stem cell transplantation in acute leukemia patients: single-centre experience

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HIGHLIGHTS

- Relapsed acute leukaemia after the first allogeneic stem cell transplantation has a poor prognosis.
- Second allogeneic transplantation may offer survival advantage for relapsed leukaemias.

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A B S T R A C T

Acute leukaemia patients who relapse after the first allogeneic stem cell transplantation (Allo-SCT) have a poor prognosis. Participating in clinical trials is the best option for these patients. If patients cannot participate in clinical trials, as the treatment options are limited, the second allo-SCT constitutes the potential curative treatment option. The data of acute leukaemia patients who underwent second allo-SCT because of relapsed/refractory disease after the first allo-SCT at our centre between December 2009 and February 2019 were analyzed retrospectively. Three hundred nineteen acute leukaemia patients were performed allo-SCT at our centre. 20 of these 319 acute leukaemia patients relapsed after first allo-SCT and underwent second allo-SCT. 10 AML patients and 10 ALL patients were included in the study. After second allo-SCT overall survival (OS) was 26.1±10.8 weeks, and progression-free survival (PFS) was 19.9±8.6 weeks. If the patients cannot participate in clinical trials, second allo-SCT should be considered for patients with late (≥12 months) relapses after the first allo-SCT. If possible, haploidentical donors should be selected for second allo-SCT and patients should be in complete remission before the transplant.

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1. INTRODUCTION

Acute leukaemias are haematological malignancies characterized by abnormal proliferation of blasts caused by hematopoietic myeloid or lymphoid precursors or both. Monoclonal hematopoietic progenitor cells lose their skills to normally differentiate and proliferate. The most frequently seen acute leukaemia type in adults is acute myeloid leukaemia (AML), and it has an incidence of 5-8 /100.000.[1,2] On the other hand, acute lymphoblastic leukaemia (ALL) has an incidence of 1.28 / 100.000, and it is less commonly observed in adults compared to AML.[3] Acute leukaemias become symptomatic in a short time due to their aggressive nature. In spite of intensive treatment methods, they have a poor prognosis. Better survivals have been achieved with improvements in intensive chemotherapies and supportive care. In addition to this, targeted therapies have been started to use in selected acute leukaemia patients. Despite these improvements and new agents in acute leukaemia treatment, the relapse rate is still high.

Allogeneic stem cell transplantation (Allo-SCT) is being used as a curative treatment method in the treatment of acute leukaemia. Some patients relapse after the first allo-SCT. The acute leukaemia patients who relapse after the first allo-SCT have poor prognoses.[4] Participating in a clinical trial is the best option for these patients. If patients cannot participate in a clinical trial, as the treatment options for these patients are limited, second allo-SCT constitutes the potential curative treatment option.[5] Some previous studies revealed that in acute leukaemia patients who relapsed after the first allo-SCT and underwent second allo-SCT, the survival was better than those of the patients who received only chemotherapy.[6,7,8] However, data indicate that only a limited number of acute leukaemia patients who relapsed after the first allo-SCT could be taken to the second allo-SCT.[6,9] Because these patients previously received multiple line therapies and generally had a bad performance.[6,9] There is still a limited number of studies regarding the efficiency of second allo-SCT and the factors impacting the survival after the second allo-SCT. The data in the literature generally come from retrospective studies conducted limited number of patients. In this study, we aimed to analyze the outcome of second allo-SCT in acute leukaemia patients who relapsed after the first allo-SCT and to find out the factors impacting the survival rates of second allo-SCT.

2. MATERIAL AND METHOD

The acute leukaemia patients who underwent the second allo-SCT due to relapsed/refractory disease after the first allo-SCT at Dr. Abdurrahman Yurtaslan Ankara Oncology Training and Research Hospital, Bone Marrow Transplantation Unit between December 2009 and February 2019 were included in the study. The local ethical committee approval received. Patients that underwent the second allo-SCT performed because of graft failure after the first allo-SCT was not included in the study. The data were analyzed retrospectively.

In acute leukaemia patients, complete response (CR) was described as having thrombocyte count ≥100.000/μL, neutrophil count ≥1000/μL in peripheric blood, blast ratio <5% in the bone marrow and having normal maturation in all hematopoietic series of bone marrow in addition to transfusion independence and not having the extramedullary disease.[10] After achieving CR, having blasts in peripheric blood or extramedullary area or having ≥20 blasts in bone marrow were defined as relapse.

In both the first and second allo-SCT, peripheric blood-derived stem cells were used. Human leukocytes antigen (HLA) typing (HLA, A, B, C, DR and DQ) was performed by high-resolution method. In second transplantations, full matched (10/10) and mismatched (9/10) siblings, relatives other than siblings, unrelated donors and haploidentical related donors were used.

The intensity of conditioning regimens was classified according to the published criteria of the Center of International Blood and Marrow Transplant Research (CIBMTR).[11] The prophylaxis of graft-versus-host disease (GVHD) was carried out with cyclosporine. Acute GVHD was graded according to the severity index of the International Bone Marrow Transplant Registry (IBMTR).[9] Chronic GVHD was graded according to the criteria of the National Institute of Health (NIH) 2015 consensus.[12]

The overall survival (OS) after allo-SCT was defined as the duration from transplant date to the date of death or to the date of the latest follow-up for the survivors. The progression-free survival (PFS) after allo-SCT was described as the duration from the date of the transplant to the first date when there was a progression or to the date of death or to the latest follow-up date for progression-free patients. The transplant-related mortality (TRM) was defined as the cumulative death within the first 100 days after allo-SCT without any evidence of disease progression. The engraftment definition for neutrophil was defined as the first day when the absolute neutrophil count (ANC) was >500/mm^3 or 1000/mm^3 for three consecutive days without any support, and for thrombocytes, it was defined as the first day when thrombocyte count was >20000/mm^3 for three consecutive days without any support.[13]

The statistical analyses were performed with IBM SPSS Statistics v21 software. Chi-square test was used for descriptive statistics and comparisons among the groups for categorized data, and Mann Whitney U tests were applied for nonparametric figurative data. Survival rates were calculated by Kaplan-Meier survival analysis and Cox regression analysis. The impacts of the variables over OS and PFS were examined using a log-rank test. The cases where Type-1 error level was under 5% was accepted as statistically significant.

3. RESULTS AND DISCUSSION

Three hundred nineteen acute leukaemia patients were performed allo-SCT at our centre. Among survivors, the median follow-up is 6.4 years. TRM is %12.4. Twenty of 319 acute leukaemia patients relapsed after first allo-SCT and underwent second allo-SCT at our centre. Ten AML patients and 10 ALL patients were included in the study. There were nine female and 11 male patients. The median age at the time of the second allo-SCT was 31 (range 18-55). The median duration between first allo-SCT and relapse was 25 weeks (range 8-219 weeks). When the patients who had relapsed within 12 months after the first allo-SCT were compared with the patients who had relapsed after 12 months, PFS after the second allo-SCT was found significantly shorter in early relapsed patients (p:0.021); however, there was no statistically significant difference regarding OS after second allo-SCT between early and late relapsed patients(p:0.102).

The median duration between the first and second allo-SCT was 50 weeks (range 14-236 weeks). While we did not find any statistically significant difference between post-transplant PFS and duration between the first and second allo-SCT (p:0.141), we found a statistically significant difference between post-transplant OS and the duration between the first and second allo-SCT (p:0.02*). Before the second allo-SCT, nine patients were full chimeric, 11 patients were mixed chimeric. In full chimeric patients, post-transplant PFS and OS were significantly longer than mix chimeric patients (p:0.013).

Second allo-SCT was performed from the donors' of 10/10 compatible siblings in 11 patients, 10/10 compatible relatives other than siblings in 2 patients, 10/10 compatible unrelated in 1 patient, 9/10 compatible unrelated in 2 patients, and haploidentical donors in 4 patients.

In second allo-SCT; 13 full matched related donors(11 siblings, two relatives other than siblings), three unrelated donors (1 full matched, two mismatched), four haploidentical donors were used. In patients whose donors were related median post-transplant PFS was 21,45 ±9,27 weeks and post-transplant OS was 25,42±13,34 weeks. In patients with unrelated donors, median post-transplant PFS was only 2.5±1 weeks, and post-transplant OS was three weeks. In patients with haploidentical donors, post-transplant OS and PFS were longer than patients with matched/miss-matched donors.

While in 11 patients' same donors were used in the second allo-SCT, different donors were used in 9 patients. We did not find any impacts of using alternative donors over PFS and OS. Myeloablative conditioning was used in 11 patients, and reduced intensive conditioning (RIC) regimen was used in 9 patients. There was no significant difference between post-transplant OS and PFS and conditioning regimen (p:0.287; p:0.265, respectively). The patients had an average of 1 cycle chemotherapy (range 1-3) in the duration between relapse and the second allo-SCT. While 13 patients were taken to the second allo-SCT with CR, seven patients were taken with active disease. There was no statistically significant difference between pre-transplant response and post-transplant PFS and OS (p:0.105 and p:0.295 respectively).

Median follow up duration was 34 weeks (range 20-257 weeks). 7 of 20 acute leukaemia patients (35%) performed second allo-SCT are still in remission. After second allo-SCT OS was 26.1±10.8 weeks and PFS was 19.9±8.6 weeks. The post-transplant PFS was 5.28±2.7 weeks, and OS were 14.88±8.65 weeks in AML patients while post-transplant PFS was 38.66±27.6 weeks, and OS was 40.4±17.1 weeks in ALL patients. There was no statistically significant difference between the patients with both diagnoses regarding the PFS and OS after second allo-SCT (p:0.059, p:0.230 respectively).

At the end of the follow-up time, nine patients died. While 3 of these deaths (15%) were related to relapse, 6 of them (30%) were related to TRM. TRM was found 20% among AML patients and 40% among ALL patients. In all patients, neutrophil engraftment occurred at a median of 14 days, and thrombocyte engraftment occurred at a median of 13 days. After the second allo-SCT, CR was observed in 9 patients (45%). Nine patients (45%) were full chimeric, 11 patients were mixed chimeric. Grade 3-4 acute GVHD was observed in 2 (10%) patients. Chronic GVHD was observed in 6 (30%) patients. The relation between post-transplant PFS and OS and diagnosis, gender, donor type, alternative donors, the presence of acute or chronic GVHD, pre-transplant response, post-transplant response, chimerism is shown in the table (Table 1).

Acute leukaemia patients relapsing after allo-SCT generally have received multiple line chemotherapies including various agents previously, and they usually show resistance to the previously used chemotherapeutic agents.[4] In such patients salvage chemotherapy, discontinuation of immunosuppression, donor lymphocyte infusion, second allo-SCT can be considered. If possible, patients should be encouraged to participate in clinical trials.

In the study conducted by Cerny et al. AML, ALL, myelodysplastic syndrome and chronic lymphocytic leukaemia patients that underwent the second allo-SCT, 1-year OS was found 44%, and the median post-transplant OS was found 8.9 months (range 0-27.6 months).[14] In the study conducted by Aljasem et al., 3-year OS rate was found 35%.[15] In another study, 1-year OS after second allo-SCT in AML patients was 20%.[16] In an analysis of CIBMTR, 1-year OS in 369 patients that underwent second allo-SCT was 49%, and the median survival was found 12 months.[17] In our study, on the other hand, the OS after the second allo-SCT was found 26.1±10.8 weeks and PFS was found 19.9±8.6 weeks, 1-year OS was 11%. In AML patients, the PFS after second allo-SCT was found 5.28±2.7 weeks, and the OS was 10.88±8.65. In ALL patients, the PFS after second allo-SCT was found 38.66±27.6 weeks and OS were found 40.4±17.1 weeks.

According to the literature, CR achievement decreases as the number of chemotherapy lines increase.[18,19] Therefore, obtaining a CR in acute leukaemia patients relapsed after the first

allo-SCT is more difficult than the second allo-SCT. In addition to this, organ functions generally impair due to high-dose treatments.[19] As a result, some of the patients are taken to second allo-SCT without having a CR. Although there are many studies conducted to increase the CR rate after relapse, the results of these studies are inadequate and more studies are required.[5,18,20,21,22] In the study conducted by Chueh et al. pre-transplant response was found to be an independent factor impacting the success of second allo-SCT.[23] In this study, 70.4% of the patients had CR before second allo-SCT. In our study, 65.2% of the patients had CR before second allo-SCT. We did not find any statistically significant difference between pre-transplant response and post-transplant PFS and OS (p:0.105 and p:0.295 respectively).

If the patients have related donors, they can use their relatives for the second allo-SCT; however, using the same donor for the second allo-SCT is relatively more difficult if the previous donors are unrelated. In the study conducted by Chueh et al., there was no difference regarding the survival rates between the patients that underwent the second allo-SCT from an alternative or the same donors.[23] In the study conducted by Aljasem et al., no relationship was observed between OS and alternative donor use.[15] The previous studies could not show the advantages of selecting an alternative donor, either.[9,19] Similarly, we did not find any statistically significant difference between the patients that underwent second allo-SCT from the same donors or alternative donors regarding post-transplant PFS and OS.

There are studies that show the positive effects of GVHD or HLA incompatibilities on the prevention of relapse. In some studies, while positive effects are observed on the rates of survival, in other benefits could not be indicated.[24,25,26] In the study conducted by Chueh et al., no relationship was found between acute or chronic GVHD observed after second allo-SCT and survival.[23] In our study, also, we did not find any statistically significant relationship between chronic GVHD and PFS and OS (p:0.737 and p:0.825 respectively); however, a statistically significant relationship was found between acute GVHD and PFS and OS (p:0.041 and p:0.029 respectively).

Some studies reported that the duration between the first allo-SCT and the second allo-SCT is an important prognostic factor, and it is related to better survival rates.[27,28] In the study conducted by Chueh, there was no statistically significant survival difference between the patients with early relapses and late relapses.[23] However in the study conducted by Aljasem et al., 3-year OS was 21% in early relapsed patients, and it was 46% in patients relapsed after 12 months (p:0.009) (20). In our study, we found shorter PFS in early relapsed patients (p:0.021), but there was no statistically significant difference regarding OS (p:0.102).

In the study conducted by Cerny et al. in second allo-SCT, median neutrophil engraftment was achieved in 15 days, and median thrombocyte engraftment was achieved in 21 days. The rate of TRM was found at 30%.[14] In our study, neutrophil engraftment occurred at a median of 14 days, and thrombocyte engraftment occurred at a median of 13 days. Furthermore, the rate of TRM was similarly found 30%. The reason for early neutrophil and thrombocyte engraftment may be peripheric blood-derived stem cells.

In the majority of second allo-SCT in the literature reduced-intensity conditioning (RIC) regimens were used.[29] In the study conducted by Aljasem et al., 3-year OS was found 42% in myeloablative regimens and 23% in non-myeloablative and RIC regimens; however, we did not find any statistically significant difference between myeloablative regimens and non-myeloablative and RIC regimens (p=0.08) (20). Similarly, in our study, no statistically significant relationship was found between OS and PFS and conditioning regimens (p:0.287; p:0.265).

4. CONCLUSION

Patients who were performed second allo-SCT had a poor prognosis. Development of new immune therapeutics with less toxicity and new strategies are required. Patients should be encouraged to participate in clinical trials. If the patients cannot participate in clinical trials, second

allo-transplant should be considered in patients who have late (≥12 months) relapses after the first allo-SCT, If possible, haploidentical donors should be selected for second allo-SCT and patients should be in CR before the transplant. More prospective studies are needed to design future treatment approaches.

DISCLOSURE STATEMENT

The authors reported no potential conflict of interest.

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Table 1. The variables that have an impact on post-transplant PFS and OS

	n	PFS after 2nd allo-SCT (weeks)	OS after 2nd allo-SCT (weeks)	P value (PFS)	P value (OS)
Gender					
Male	11	8,83 ± 3,72	14,33 ±8,41	0,386	0,824
Female	9	31 ±16,2	31,66 ± 30,67		
Diagnosis					
AML	10	5,28± 2,7	10,88±8,65	0,059	0,230
ALL	10	38,66 ±27,6	40,4 ± 17,1		
Donor Type					
Same donor	11	9,71 ± 3,44	14±8,5	0,270	0,518
Alternative donor	9	32,33±30,35	34,2 ± 19,38		
Donor Type					
Related donor	17	21,45 ±9,27	25,42±13,34	0,297	0,068
Unrelated donor	3	2,5±1,5	3		
HLA Typing					
HLA full matched	14	17,625±8,16	16,2±10,06	**0,034***	0,112
HLA mismatched	2	1,5±1,5	1,5 ±1,5		
Haploidentical	4	47,5±43,5	48,5±44,5		
Acute GVHD (grade 3-4)					
Yes	7	45,75±21,195	73,5± 19,5	**0,041***	**0,029***
No	13	4,85 ±2,58	7±2,56		
Chronic GVHD					
Yes	6	31±30	31,66± 30,67	0,737	0,824
No	14	14,33± 8,41	16,2 ± 7,33		
Pre-transplant Response					
CR	13	28,12 ± 12,057	28,66±15,33	0,105	0,295
Refractory	7	3±0,57	3,5 ± 0,64		
Relapse after 1st allo-SCT					
Early relapse	3	7,7 ± 2,6	11±6,53	**0,021***	0,102
Late relapse (≥12 months)	2	81 ± 10	93+0		
Post-transplant Response					
CR	9	44±15,44	55,66 ±21,1	**0,002***	**0,018***
Refractory	11	2± 0,9	2,6±0,92		
Chimerism					
Full	9	50 ± 18,37	55,66 + 21,1	**0,046***	**0,013***
Mix	11	2,3 ± 0,66	5		

AML: acute myeloid leukemia, ALL: acute lymphoblastic leukemia, HLA: human leukocyte antigen, CR: complete response, GVHD: graft versus host disease, PFS: progression free survival, OS: overall survival

SHORT BIOGRAPHY

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He was born in Köyceğiz, Turkey. He graduated from Black Sea Technical University, Medical Faculty in 2011. Between 2012 and 2016, he worked as a Research Assistant at Internal Medicine of Mediterranean University Faculty of Medicine. He worked in Van State Hospital in 2017 as an Internal Medicine specialist. He has been working in Ankara Oncology Training and Research Hospital, Hematology Service and Bone Marrow Transplant Unit as an Hematology fellow since 2017. He has many articles published in international SCI and national journals.

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Original Research

Does platelet/ lymphocyte ratio a predictor of CD34+ peripheral blood stem cell yield in the healthy donors mobilized with GCSF?

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HIGHLIGHTS

Consequently, a high platelet/lymphocyte ratio may be correlated with the number of collected CD34+ stem cells

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A B S T R A C T

This research aimed to determine whether there is a correlation between platelet/lymphocyte ratio the number of collected CD34+ stem cells. This retrospective study included 94 adult related stem cell donors who were healthy and volunteer by screening their files between the years 2016 and 2018. All donors were mobilized using 2.5 mcg/kg lenograstim or filgrastim and underwent peripheral stem cell apheresis. Complete blood counts were tested at baseline before G-CSF administration (pre–G-CSF), and before PBSC collection after mobilization with G-CSF administration. The patients were divided into two groups as aged below and over 50 years old. From these comparative data, only BMI value of the group aged below 50 years was statistically significantly lower than the other group, whereas no statistically significant difference was found between the groups in terms of other parameters. The numbers of the collected CD34+ stem cells of both age groups were similar, and no significant difference was found. The values of platelet/lymphocyte ratio, early measurement of CD34+ stem cells and the amount of the collected CD34+ stem cells of both groups at the first and second days of the procedure were found similar. This research show that a high platelet count and consequently, a high platelet/lymphocyte ratio may be correlated with the number of collected CD34+ stem cells but our hypotheses revealed insignificant outcomes.

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1. INTRODUCTION

Peripheral blood stem cell collection for allogeneic or autologous stem cell transplantation has become the standard treatment modality thanks to easy-applicability and rapid haematological reconstitution compared with stem cell collection from bone marrow harvest in many malignant and benign cases.[1,2] Stem cell mobilization is performed with granulocyte-colony stimulating factor (GCSF) in healthy donors. The donor response to G-CSF influences the number of CD34+ cells and eventually transplantation outcomes. Low level of peripheral blood stem cell increases the risk for graft failure.[3] On the other hand, transplantation of high CD34+ stem cell doses increases the risk for acute graft versus host disease (GVHD).[4] Nevertheless, a high level of stem cell infusion in the patient is considered to be associated with faster immune reconstruction.[5] As known, GCSF causes lymphocytosis, however, GCSF has several effects on T-cell subgroups such as especially mobilization of naive T lymphocytes, reduced adhesion molecules and chemokine receptors, reduced T-helper-1-related cytokines and increased regulatory T (T-reg) cells.[6] It is also known that GCSF suppresses pro-inflammatory cytokines and increases secretion of IL-10. All these effects indicate the immunosuppressive nature of GCSF in healthy humans.[7] On the other hand, it has been shown that platelet count in the peripheral circulation decreases due to G-CSF administration.[8] The reduction in platelet count is directly proportional with GCSF dose.[9] Baseline platelet counts prior to the onset of GCSF administration is strongly correlated with the amount of mobilized CD34+ stem cells.[10,11]

In the light of all these information from the literature, we have retrospectively evaluated whether there is a correlation between the platelet/lymphocyte ratio as a cheap and easy-applicable method and the number of collected CD34+ stem cells.

2. MATERIAL AND METHOD

This retrospective study included 94 adult related stem cell donors who were healthy and volunteer by screening their files in the Bone Marrow Transplant Unit of Medicana Ankara International Hospital between the years 2016 and 2018 (Ethics Committee Approval Dated 08.02.2019, Number:26). All donors were over 18 years old. A haematologist from the bone marrow transplant team approved the donors by evaluating their detailed anamnesis, physical examination, complete blood count, biochemistry and infection markers, blood grouping and Rh typing, and pregnancy testing in the females of childbearing age. The donors with a chronic disease and ongoing medication were excluded from the study. Body mass index (BMI) was calculated according to the formula BMI= weight (in kilograms)/height (in meters squared). All donors were mobilized using 2.5 mcg/kg lenograstim or filgrastim and underwent peripheral stem cell apheresis.

The onset of G-CSF administration was accepted as the 1st day, and the donors underwent mobilization procedure with Fresenius (Bad Homburg, Germany) device if the early measurement of CD34+stem cells resulted in $20x10^6$ /L at the 5th day. The collection of minimum $2x10^6$ /kg body weight CD34+ stem cells was targeted. The donors underwent apheresis procedure for 1 or 2 days according to that target cell dose or transplant planning of the patient. The blood volumes were processed for 2-4 times and coagulated with 40-90 mL/minute acid-citrate dextrose as the coagulant in the healthy donors (HD) determined to be performed the procedure at the second day. The healthy donors (HD) were administered 10% Calcium Gluconate. Complete blood counts were tested at baseline before G-CSF administration (pre–G-CSF), and before PBSC collection after mobilization with G-CSF administration.

The parameters were analyzed by SPSS for Windows Version 23.0. The continuous variables were expressed as median (minimum-maximum); numbers while percentages were used to express categorical variables. The differences between continuous variables of two groups were calculated by Mann-Whitney-U test. The repeated measures were compared by Wilcoxon test. Chi-square test was used in the analysis of categorical parameters. Spearman Rho

correlation was used to calculate the correlation of two continuous variables. The $p<0.05$ value was accepted as the statistical significance level.

3. RESULTS AND DISCUSSION

Totally 94 donors were included in the study. The study group consisted of 38 females and 56 males. The patients were divided into two groups as aged below and over 50 years old. This distribution was based on the consideration that the platelet/lymphocyte ratio may have an impact on the amount of CD34+ stem cells depending on donor age. Median haemoglobin (Hg) value was 15 g/dl in the group aged below 50 years, whereas that value was 14.7 g/dl in the group aged ≥50 years. WBC counts of the groups were found $7.08x10^3$/mL and $7.24x10^3$/mL, respectively. Body mass index (BMI) value of the younger group was 24.9, whereas that value was 27.8 in the group aged ≥50 years (Table 1). From these comparative data, only BMI value of the group aged below 50 years was statistically significantly lower than the other group, whereas no statistically significant difference was found between the groups in terms of other parameters. The numbers of the collected CD34+ stem cells of both age groups were similar, and no significant difference was found (Table 2). In addition, we have analyzed the platelet/lymphocyte counts in two groups as <100 and >100 (Table 3). The values of platelet/lymphocyte ratio, early measurement of CD34+ stem cells and the amount of the collected CD34+ stem cells of both groups at the first and second days of the procedure were found similar.

In the present study, we have analyzed the relationship between baseline platelet/lymphocyte ratio and the amount of CD34+ stem cells collected following mobilization. The previous studies have suggested assumptions on predicting a number of the CD34+ stem cells based on various demographic and laboratory analyses and reported that the most effective factors on CD34+ stem cell apheresis yield are G-CSF dose administered in the donor as well as age, gender and the bodyweight of the donor,[10] however, no study on platelet/lymphocyte ratio has been conducted yet as far as we know. It has been aimed to predict the amount of CD34+ stem cells that can be collected by performing an easy and low-cost method in this study.

The peripheral blood stem cell collection from healthy donors induced with GCSF is an easy technique with a low incidence of complication. The amount of the collected CD34+ stem cells has various effects on engraftment. The detection of these effects in the early pretransplantation period is important in the regulation and prediction of the posttransplantation period.

It is known that a high level of baseline platelet count before GCSF administration is proportional to the number of mobilized CD34+ stem cells.[10,11,12] In the present study, we did not analyze the relationship of platelet count with baseline value before GCSF administration and number of the CD34+ stem cells before the procedure. In addition, some studies have suggested that the amount of the collected stem cells may decrease with advancing age, whereas some other studies assert the contrary to this conclusion.[13] Various studies on donor age have presented different outcomes.[14,15] De La Rubia et al. have reported that a higher number of CD34+ stem cells was collected in the younger donors. From this point of view, we distributed our donors into two groups as ones aged below and over 50 years old. We have determined that the platelet/lymphocyte ratio was not significantly correlated with the amount of CD34+ stem cells in both groups.

Our study has some limitations. As our study is retrospective, we could not obtain lymphocyte subgroups and also it would be better if we could search interleukins, tumour necrosis factors, and interferon-alpha because these mediators are effected by GCSF also, if we could not use one type of generic for all donors we had to use filgrastim or lenograstim both.

4. CONCLUSION

As an overall conclusion, we have assumed that a high platelet count and consequently a high platelet/lymphocyte ratio may be correlated with the number of collected CD34+ stem cells or contrarily a platelet/lymphocyte ratio encountered below a certain threshold value due to extremely reduced platelet count after GCSF administration may also be proportional with the collected CD34+ stem cells in developing hypotheses of the study. However, both of our hypotheses revealed insignificant outcomes.

DISCLOSURE STATEMENT

The authors reported no potential conflict of interest.

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Table 1. The demographic characteristics of the donors

	Age Group		p
	<50 of age (n=54)	≥50 of age (n=40)	
	Median (min-max) / n (%)		
Gender			0.724**
Female	21 (38.9%)	17 (42.5%)	
Male	33 (61.1%)	23 (57.5%)	
Hgb (mg/dL)	15 (9.5-17.3)	14.7 (11.3-16.7)	0.857*
WBC (x10^3/µL)	7.08 (4.02-11.7)	7.24 (4.39-11.3)	0.731*
Weight (kg)	73.5 (50-102)	79 (50-107)	0.096*
Height (cm)	170 (153-188)	169 (148-180)	**0.016***
BMI (kg/m^2)	24.94 (18.94-35.71)	27.8 (20.52-40.27)	**0.001***

*Mann Whitney-U test. ** Chi-square test

Table 2. Efficacy and effect of HSC mobilization according to age group of the donors

	Age Group		p*
	<50 of age (n=54)	≥50 of age (n=40)	
	median (min-max) / n(%)		
Apheresis number			0.320***
First	45 (83.3%)	30 (75.0%)	
Second	9 (16.7%)	10 (25.0%)	
Blood volume processed (mL)			
First	350 (200-455)	341.5 (230-407)	0.319
Second	280 (230-377)	323 (220-391)	0.347
Difference between first and second **p****	**0.038**	0.123	
CD34$^+$ cell apheresis day 1 and 2 (10^6/L)			
First	61 (19-284)	60.5 (10-138)	0.731
Second	39 (27-64)	27.5 (5-121)	0.086
Difference between first and second **p****	0.859	0.066	
CD34 (yield/kg)			
First	4.56 (1.7-19.01)	4.25 (1.08-10.99)	0.244
Second	2.23 (1.32-3.33)	1.78 (0.83-12.73)	0.221
Total	4.85 (1.71-19.01)	4.49 (1.71-15.75)	0.304
Difference between first and second **p****	0.260	0.074	
Lymphocyte			
First	2,42 (1,28-4,39)	2,33 (1,15-4,14)	0,328
Second	3,91 (0-7,8)	3,89 (1,71-5,64)	0,745
Platelets			
First	242 (164-483)	228 (114-354)	0,309
Second	203 (114-339)	180 (79,4-356)	0,152
Platlet/lymphocyte ratio (PLO)			
First	101,76 (54,37-206,77)	98,21 (51,68-184,35)	0,722
Second	49,38 (0-134,72)	48,82 (19,93-82,41)	0,994

*Mann Whitney-U test. ** Wilcoxon test. *** Chi-square test

Table 3. Platelet lymphocyte ratio comparisons

	PLO_1		p	PLO_2		p
	<100	**≥100**		**<100**	**≥100**	
CD34 count (apheresis day 1)	61 (10-215)	60.5 (19-284)	0.967	61 (10-284)	37 (37-37)	0.246
CD34 count (apheresis day 2)	24 (5-55)	38 (16-121)	0.272	38 (5-121)	28 (28-28)	0.584
CD34_kg_1	4.41 (1.08-18.3)	4.32 (1.64-19.01)	0.487	4.4 (1.08-19.01)	2.75 (2.75-2.75)	0.261
CD34_kg_2	1.88 (0.83-3.33)	2.12 (1.32-12.73)	0.353	2 (0.83-12.73)	2.54 (2.54-2.54)	0.465
CD34_kg_T	4.67 (1.98-18.3)	4.65 (1.71-19.01)	0.800	4.66 (1.71-19.01)	5.29 (5.29-5.29)	0.699

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Original Research

The potency of green grass jelly extract (Premna oblongifolia Merr) as antihyperlipidemia towards aorta histopathology representation of rat (Rattus norvegicus) induced with high fatty diet (HFD)

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HIGHLIGHTS

Green grass jelly extract (*Premna oblongifolia Merr)* could prevent fatty acid cell infiltration and prevent macrophag infiltration in rat (*Rattus norvegicus*) hyperlipidemia model

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A B S T R A C T

Green grass jelly *(Premna oblongifolia merr)* is a plant containing fiber and chlorophyll which can lower cholesterol and triglyceride levels. This study have an aim to investigate the potency of green grass jelly extract *(Premna oblongifolia Merr)* to prevent hyperlipidemia. The animal mode used for this study is male *Rattus norvegicus*, Wistar strain, the age of 8 weeks, and weight of 200 g which is divided into 5 groups of treatment namely group Kn (negative control), Kp (positive control), Kp1, Kp2, and Kp3 induced with HFD and green grass jelly extract at a dose of 5.27 g/ kg BW/ daily, 8.43 g/ kg BW/ daily, 9.37 g/ kg BW/ daily. The data of infiltrasi fatty cells and makrophag on aorta histopatholgy was analyzed by description. This research showed that treatment of green grass jelly extract *(Premna oblongifolia Merr)* to animal of hyperlipidemia model reduced infiltration fatty cells and makrophag. The conclusion of this study was the green grass jelly extract was able to prevent increase of fatty cells and makropha infiltration of rat *(Rattus noervegicus)* induced with HFD on dose 9,37 g/ kg/ BW/ daily.

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1. INTRODUCTION

Hyperlipidemia is a condition of increase blood lipid level consist cholesterol, triglyceride, and *LDL level (low-density lipoprotein)* but decreases blood lipid-like *HDL level (high-density lipoprotein)*. HDL level normal on blood plasma Rattus ≥35 mg/dL, the normal LDL level is 7-27.2mg/dl, and the normal cholesterol level is 10-54mg/dl.[1]

Hyperlipidemia could happen on pets (cat and dog). Chronic hyperlipidemia could cause endothelial dysfunction. The endothelial dysfunction caused by blood vessels increased, which make LDL easy to include at the blood vessel. Stress oxidation cause LDL easier to change to be LDL-ox, and than the macrophage scavenger receptor will catch it and change to be a foam cell. The accumulation of LDL on blood vessel walls could be cell foam.[2]

The high fatty diet was the food which compounds fat, and it can make the stress oxidation inside of the body.[2] Stress oxidative caused by free radical which binding with another compound to make a stable compound and could destroy another macromolecule, like the cell membrane of lipid, DNA and protein.[3] It could increase blood lipid, which is cholesterol and triglyceride level, which called hyperlipidemia.[4] Free radical can be caused LDL increased in the blood and also inflammation in the wall of the blood vessel. It's an infiltration of fatty cells and macrophage at tunica adventitia. The inflammation stimulated cell immunocompetent which is lymphocyte, neutrophil, monocyte and macrophage.[5]

The therapy was already used to patient hyperlipidemia, usually consist of SSRI (selective serotonin reactive inhibitor). But, a therapy used synthetic medicine has a negative effect and cause systemic distribution, vital organs like liver and ren. Green grass jelly *(Premna oblongifolia Merr)* is safe and easy therapy as preventive to hyperlipidemia. It is a plant consists of soluble fiber like pectin.[6] Because it, green grass jelly, called soluble fiber, which could decrease total cholesterol levels and serum LDL. Pectin is soluble fiber at the digestion tract, and it's used to bind bile acid. It's a final result of cholesterol and will be the end of the metabolism process as feces. It's expected could be to decrease the cholesterol level in the body.[5] This study has a purpose in knowing the fatty cell infiltration and the macrophage infiltration on aorta histopathology representation of rat hyperlipidemia model after giving preventive therapy by green grass jelly extract.

2. MATERIALS AND METHOD

Preparation Experimental number 421-KEP-UB Animal

Kn group is rat without giving any treatment (negative control/Kn), Kp group is ratt with hyperlipidemia condition (positive control /Kp), treatment group 1 (P1) is ratt with green grass jelly extract 5.27 gram/kg BW/day as preventive treatment and diet HFD *(high Fatty Diet)*, treatment group 2 (P2) is ratt with green grass jelly extract 8.43 gram/kg BW/day as preventive treatment and diet HFD *(high Fatty Diet)*, treatment group 3 (P3) is ratt with green grass jelly extract 9.37 gram/kg BW/day as preventive treatment and diet HFD. First, we have to adaptation the animal trial with laboratory area for seven days by giving them standard food to all rat. The ingredients of standard food are carbohydrate, protein, fat, mineral, vitamin, and water.

Preparation of Green Grass Jelly Extract

The sample we use is green grass jelly *(Premna oblongifolia merr)* which harvest at April 2018 in the morning in Batu, Malang. The green grass jelly we get, we cleaned it and drained it at the oven with temperature 30˚C. The green grass jelly extract then weighed with three dose different which is 1.35 g then dissolved with 8mL water (aquades), 2.7 g then dissolved with 10 mL water and 5.4 g then dissolved with 18 ml water and then just let stand every extract at bowl one by one, then homogenous it or stir it, then pour it to filter and press it, then collect the filtrate. Take the filtrate use spuit 3 mL until getting the green grass jelly with contains soluble in a small volume.

Preparation Animal Model Hyperlipidemia

Rat (*Rattus norvegicus*) can use be an animal model of obesity is male rat strain Wistar, 8 weeks, 200-gram body weight, with one-week acclimatization in the laboratory.[7] The researcher was chosen ratt (*Rattus norvegicus*) strain Wistar by easy to get, easy to care and have a fast metabolic. That was a benefit in experimental research which involved with metabolism.[8] Rat 6-8 weeks still not effected by growth hormone and sexual hormone.[9]

The male Wistar Rat, 20 rats, which is age 8 weeks, was adaptation one week at the laboratory. *High fatty diet* was compound by 1 gram quail yolk egg: 2 gram margarin: 2 fat cow.[10] A week after food adaptation, we give green grass jelly extract as preventive as the group treatment in

a week, for making sure the animal model not on hyperlipidemia phase we did total check cholesterol. Then we did the next step of treatment like give a high-fat diet an hour after green grass jelly extract treatment as long two weeks.

After that, the rat didn't give food and water (fasting) a day for did a checkup total cholesterol level. After that, to make sure the animal model already on the hyperlipidemia phase, then we need to check the cholesterol level in animal model hyperlipidemia. The result of the cholesterol level will be higher than in normal conditions.

The Green Grass Jelly Extract Treatment

The green grass jelly extract treatment starts giving after adaptation, giving by oral used sonde method to gastric rat as long 21 days. The green grass jelly extract *(Premna oblongifolia Merr)* was giving to rat with 3 concentration different which isKn group (negative control) normal rat, Kp group (positive control)rat with hyperlipidemia not give any treatment, P1 treatment (treatment 1)each rat with average weight 200 gram with 5.27 g/kg BW dose (2.5 mL), groupP2 (treatment 2) the rat with average weight 200 gram giving 8.43 g/kg BW (2.5mL) dose and group P3 (treatment 3) the rat with average weight 200 gram giving 9.37 g/kg BW (2.5mL) dose once a day.

The Analysis Histopathology Aorta Representation use H and E staining

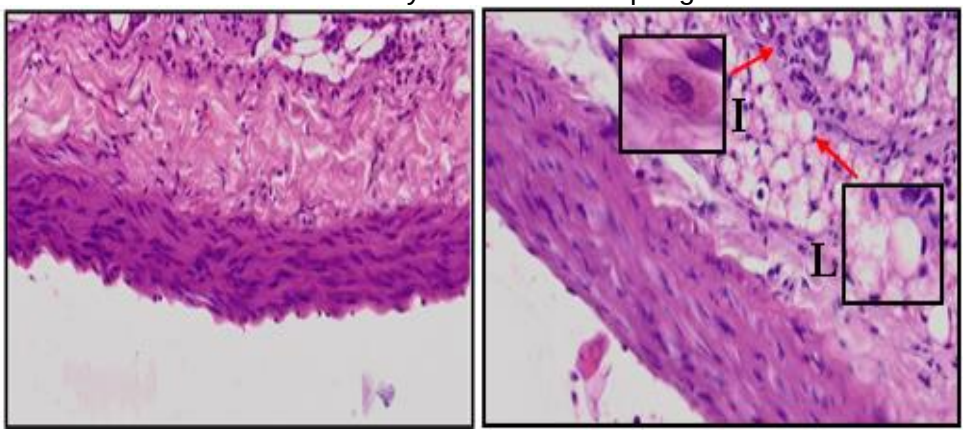
The analysis of histopathology aorta representative uses the H&E staining method by collecting the sample use PFA 4%. Then dehydration the aorta use ethanol, then make it in a paraffin block. The aorta ready to coloring use H&E staining. The next step observed it use a microscope with a 400x scale.

Analysis of Data

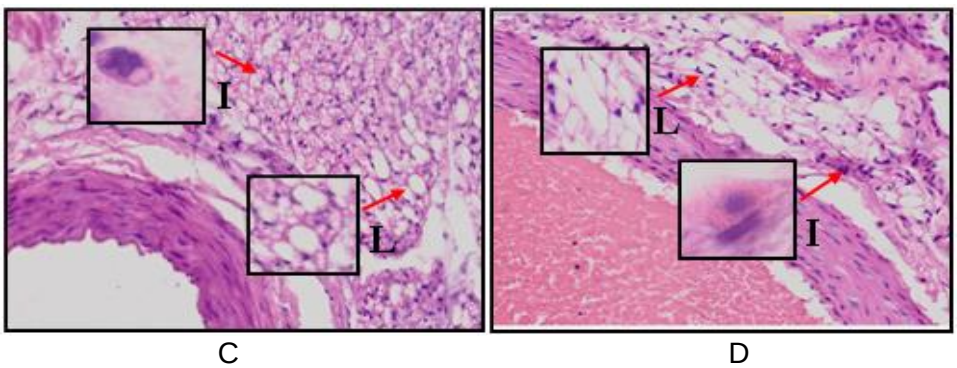
The variable we take in this research is fatty cell infiltration and macrophage infiltration. The analysis of aorta histopathology representation could do use a microscope with a 400x scale for seeing there are or absent fatty cells and macrophage infiltration. The data we get would be written by description.

3. RESULTS AND DISCUSSION

The research result of aorta histopathology representation uses a microscope with a 400x scale for observation of the infiltration of fatty acid and macrophage in the tunica adventitia.



A B



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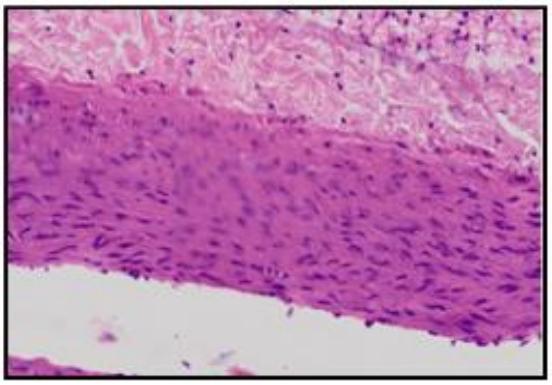

E

Figure 1. Rat *(Rattus norvegicus)* aorta histopathology representation used HE colouring with a 400x scale.
Noted:
Picture A is negative control group
Picture B is positive control group
Picture C is treatment group by green grass jelly extract 5.27 g/kg BW/ day dose
Picture D is treatment group by green grass jelly extract 8.43 g/kg BW/ day dose
Picture E is treatment group by green grass jelly extract 9.37 g/kg BW/ day dose
I is: inflammation (macrophage cell)
L is: Lipid cell

Green grass jelly extract *(Premna oblongifolia Merr)* is a plan to have fiber. The extract from this can be a gel because of the compound by water-soluble fiber. It was like pectin polysaccharide.[11] Pectin is one of some species which water-soluble food and easy to fermented by intestine microflora. Because the compound of pectin inside is a good fiber source.[12]

Stress oxidation caused by the increase of ROS as effected from HFD. Free radical incline to binding another compound to make a new compound which more stable and could destroy macromolecule, which is cell membrane of lipid, DNA, and protein, which could be stress oxidative.[13] It caused lipid peroxidation.[14] The peroxidation lipid on cell membrane-like free radical reaction with PUFA.[15] It can produce malondialdehyde (MDA).[16]

The increase of ROS caused by synthesis bile duct acid, which increased by high cholesterol and it could increase free radicals.[17] It because adipocyte and preadipocyte have identification as a source of inflammation cytokine, which is TNF-α, IL-1, dan IL-6. Cytokine is a stimulator for produce ROS and nitrogen by makrophage and monochyte. Stress oxidative is not a balance condition of oxidant and antioxidant in the body, so it could induced lipid peroxidation. Lipid peroxidation is a sequence reaction which has a signal by adding a hydrogen atom process by oxygen radical or reaction by free radical with PUFA in the cell membrane.[18] Stress oxidative was sign by MDA level, so MDA level used by the biology mark or measure of oxidative stress in the body.[19] It appropriate with the increased of infiltration lipid in the aorta which shows oxidative damage in lipid (LDL oxidation to LDLox).[20]

The aorta histopathology or rat *(Rattus norvegicus)* on figure 1.A (negative group) showed normal conditions in tunica intima. It's a layer of the aorta which direct contact with blood. This layer build by endothelial cells, the layer which closed with this layer is tunica media. Tunica media was building by smooth muscles and elastic tissue. The outer layer is tunica adventitia which is compound by connective tissue.[7]

There is three-step of lipid oxidation, which are initiation, propagation, and termination. Initiation is free lipid radical, and it is the mean compound of fatty acid, which not stabile and very reactive caused by loose one hydrogen atom. Propagation, loosed of the hydrogen atom, involved binding rearranges to stabilized with establishment Deina conjugation. Termination step showed that same radical could be fuse make a new molecule which not reactive or make a reaction with another antioxidant.[21] On that condition could make an accumulation of LDL in the blood vessel and being dysfunction endothelial. The dysfunction endothelial could make monocyte in the blood come into tunica adventitia and being lipid depotition.[22] It could be inflammation in the blood vessels and in tunica adventitia full of inflammation cells. The inflammation reaction could be caused by the initiation of immunocompetent cells like macrophage.[5] From figure 1.B could see that aorta histopathology representation clearly is infiltration macrophage and lipid cell. The trigger of influx and migration cells

didn't know clearly, and tunica adventitia looks like a point place of fatty acid cell infiltration and other inflammation cells.

The damage of endothelial cells was stimulated by ROS, which could be binding with LDL in the blood and be an LDL oxidation. That condition makes leucocyte inside of blood vessels or in the endothelial site, which damages.[23] On the other side, endothelial damage was caused by endothelial permeability to increase and be a cavity between the cells and be a lipid infiltration and inflammation cells in the tunica adventitia. It could be dysfunction endothelial or vasodilatation, which caused damage to endothelial cells.[24] But, at that histopathology, representation didn't show endothelial dysfunction clearly.

The infiltration decreased by green grass jelly extract which compound by water-soluble fiber like pectin which caused fermented easily by intestine microflora which produced acetic acid, propionate and butyrate which could inhibit cholesterol synthetic that showed on figure 1.C and 1.D.[25] The highest decreased cholesterol and triglyceride was shown on figure 1.E, it because the soluble water fiber was done by increased free radical and cholesterol with bile duct acid which in by *hepatic duct* over by pancreas, then into proximal duodenum which near with pylorus in gastritis tract and out by the last product of secretion.[20] Except for compound by green grass, fiber is also compound by antioxidants, which is chlorophyll. The high chlorophyll level could decrease cholesterol level, and triglyceride to increased, caused by chlorophyll can give oxygen which can be neutralized fee radical and inhibit free radical activation, so didn't be a stress oxidative and membrane cell damage which mark by decreased of lipid infiltraion.[15]

4. CONCLUSION

Giving green grass jelly extract (*Premna oblongifolia Merr)* with 5.27 g/kg BW, 8.43 g/kg BW dan 9.37 g/kg BW dose in rat (*Rattus norvegicus*) hyperlipidemia model could prevent fatty acid cell infiltration and prevent macrophage infiltration by giving the best dose 9,37 g/kg BW.

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CONFLICT OF INTEREST

We declared in this work, not any conflict of interest.

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Original Research

The potential of traditional balinese spices against the growth of Salmonella sp in vitro

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HIGHLIGHTS

Traditional Balinese have antimicrobial potential.

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A B S T R A C T

Prevention by utilizing medicinal plants can be used as an alternative treatment. This study aims to identify active antimicrobial substances and test the antimicrobial potential of traditional Balinese spices, namely Basa Selem, Basa Gede, Basa Wangen, Basa Bawang Jahe, and Basa Rajang against *Salmonella sp* bacteria *in vitro*. The True-experimental method with Posttest only-control design was used in this study, by intervening in the treatment group as well as the presence of positive and negative controls. Maceration method with 96% EtOH solvent was used to extract active substances and identify the levels of antimicrobial active substances. The TLC Spectrophotodensitometer instrument and the diffusion method (discs) were used to test antimicrobial potential. Data analysis was performed using the one-way ANOVA test. All five samples showed flavonoids, alkaloids, tannins and phenols in qualitative tests. Based on quantitative test results of five samples, the highest compound content obtained in Basa Wangen (6.66 mg/ml of tannins), Basa Gede (3.74 mg/ml of flavonoids), Basa Bawang Jahe (2.49 mg/ml of tannins), Basa Selem (2.87 mg/ml of tannin), and Basa Rajang (6.96 mg/ml of flavonoids). There are differences in the antimicrobial potential of various types of traditional Balinese spices against the growth of *Salmonella sp* in vitro (sig = 0.037). The traditional Balinese spices have the antimicrobial potential of the intermediate category with a range of inhibition (16-20 mm) based on the NCCLS standard.

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1. INTRODUCTION

Indonesia has a diversity of cultures along with the development of various types of food with diverse flavours as well. Spices are often used in various culinary regions in Indonesia as a cooking ingredient. In addition to increasing food flavour, added spices can also increase product durability and also product safety for consumption. Increased shelf life and safety of food products added to spices are caused by the ability of spices to inhibit the growth of bacteria present in food.[1] Many spices are also synergists when combined, and those spices exhibit greater antibacterial effects than when each is used alone.[2]

Spices are also believed to be beneficial for health because they contain many bioactive compounds that function as antimicrobials, antioxidants, antidiabetic, antitumor, and other functions that are very beneficial for maintaining health. For this reason, spices are widely developed for traditional medicine because they are believed not to cause harmful side effects[1]. The food ingredients made from spices are processed in a simple way. Traditional spices are very beneficial in the absence of chemicals such as monosodium glutamate (MSG), which are commonly used as flavouring ingredients.[3] The use of traditional spices which is a collection of various spices in making Balinese sausages (Urutan) functions as a flavouring, preservative, antioxidant and antimicrobial .[3]

The content of essential oils in spices can be used to inhibit the growth of bacteria in food. The results of other studies show that spices produce Allicin (the active substance of garlic) can penetrate quickly into different parts of the cell and cause biological effects.[1] Other ingredients that are often used in traditional herbs are garlic, mustard, cloves and ginger. Cloves have the highest antibacterial activity followed by garlic, while mustard and ginger show smaller antibacterial activity.[1] The spices are also found in traditional Balinese spices such as Basa Wangen, Basa Gede, Basa Bawang Jahe, Basa Selem, and Basa Rajang. This research was conducted to study the antimicrobial potential of traditional Balinese spices against the growth of *Salmonella sp* in vitro.

2. MATERIAL AND METHOD

There are five types of traditional Balinese spices such as Basa Wangen, Basa Gede, Basa Bawang Jahe, Basa Selem, and Basa Rajang. The total number of samples from this study was 56 samples, where there were eight times replications from 5 treatment groups with one positive control and one negative control[4]. Antimicrobial potential was evaluated by measuring inhibition zone diameters, using 96% EtOH as a negative control and Ciprofloxacin 5 µg as a positive control. Maceration method with 96% EtOH solvent was used to extract active substances and identify the levels of antimicrobial active substances. The TLC Spectrophotodensitometer instrument and the diffusion method (discs) were used to test antimicrobial potential. The True-experimental method with Posttest only-control design was used in this study, by intervening in the treatment group as well as the presence of positive and negative controls. Data analysis began with the Kolmogorov Smirnov test, then One Way ANOVA (Analysis of Variance) test was used to determine the inhibition zone of various types of traditional Balinese spices extract, then compared to the NCCLS table (National Committee for Clinical Laboratory Standards) to determine the most potential types of traditional Balinese spices as antimicrobials against the growth of *Salmonella sp*.

3. RESULTS AND DISCUSSION

The results of qualitative tests by phytochemical screening on the content of antibacterial compounds of traditional Balinese spices obtained the following results (Table 1).

Table 1. Qualitative Test Results of Antibacterial Compounds from Traditional Balinese Spices Extracts

No	Spices Samples	Antibacterial Compounds Test Results				
		Alkaloids	Saponin	Phenol	Flavonoids	Tannins
1	Basa Wangen	+	-	+	+	+
2	Basa Gede	+	-	+	+	+
3	Basa Bawang Jahe	+	-	+	+	+
4	Basa Selem	-	-	+	+	+
5	Basa Rajang	-	-	+	+	+

Noted: (+) detected; (-) not detected

Qualitative test results indicate there is a positive compound; it is suspected that the compound has antibacterial activity (phenol, flavonoid, and tannin compounds). Quantitative tests were continued to determine the levels of compounds suspected to have antibacterial activity (total phenols, flavonoids and tannins) (table 2).

Table 2. Quantitative Test Results of Traditional Balinese Spices Extracts Antibacterial Compounds

No	Spices Samples	Antibacterial Compounds		
		Phenol (mg/mL.GAE)	Flavonoids (mg/mL.RE)	Tannins mg/mL.GAE)
1	Basa Wangen	3.39	3.27	6.66
2	Basa Gede	1.83	3.74	1.35
3	Basa Bawang Jahe	2.14	2.46	2.49
4	Basa Selem	2.42	1.96	2.87
5	Basa Rajang	3.35	6.96	2.35

Noted: GAE: Gallic Acid Equivalents; RE: Rutin Equivalents

In this study, the inhibitory test of Balinese traditional spices concentrations of 100% (1 mg/ml) was carried out on the growth of *Salmonella sp*. bacteria using 96% EtOH as a negative control and Ciprofloxacin 5 µg as a positive control. The positive control showed a zone of inhibition until the eighth replication. The positive zone inhibition zone values obtained were not very different. The mean inhibition zone diameter obtained was 31.88 mm (Table 3).

Table 3. Inhibition Zone of Traditional Balinese Spices on the growth of *Salmonella sp.* in Vitro

Replications	Inhibition zone						
	Basa Selem (1 mg/ml)	Basa Gede (1 mg/ml)	Basa Wangen (1 mg/ml)	Basa Bawang Jahe (1 mg/ml)	Basa Rajang (1 mg/ml)	Positive control	Negative control
1	20	20	21	17	22	32	0
2	15	21	18	18	19	31	0
3	20	19	19	17	17	33	0
4	14	18	16	15	19	32	0
5	15	21	16	15	18	34	0
6	18	19	18	19	18	30	0
7	16	18	21	19	19	32	0
8	19	20	19	18	21	31	0
Mean ± SD	17.13 ± 2.42	19.5 ± 1.19	18.5 ± 1.92	17.25 ± 1.58	19.13 ± 1.57	31.88 ± 1.25	0.0 0.00

Noted: SD: Standard Deviation

The mean diameter of negative controls showed there was none inhibitory activity of *Salmonella sp*. bacteria growth, starting from replication one to replication eight, the inhibition zone diameter of negative control obtained was 0 mm.

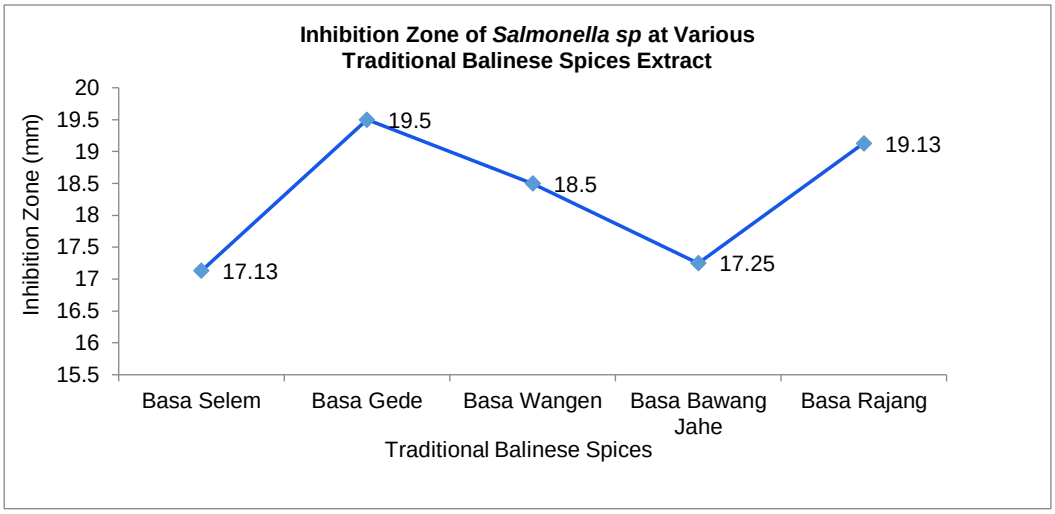

Figure 1. Inhibition Zone Differences of Various Traditional Balinese Spices Extract

Based on the results of the current study, none of the traditional Balinese spices was effective against the growth of *Salmonella sp* (when compared to the NCCLS table). Inhibitory zone diameters formed in various traditional Balinese spices are in the intermediate category (16-20 mm). One Way ANOVA test results (using software SPSS 16.00) obtained sig value was 0.037 (<0.05). The results showed there was a differences inhibition zone of *Salmonella sp* in various Balinese traditional spices with concentration 1 mg/ml. Based on these results, there are differences in the antimicrobial potential of various types of traditional Balinese spices extracts against *Salmonella sp.* The differences in inhibitory activity from various types of traditional Balinese spices extracts was analyzed by LSD (Least Significant Difference) test. The LSD test showed that p-value was 0.000 ($p<\alpha$); there were significant differences in the effectiveness of biodesinfectant at various traditional Balinese spices extracts against *Salmonella sp.*

The inhibitory test was conducted to determine the antibacterial potential of traditional Balinese spices against *Salmonella sp*. bacteria. Traditional Balinese spices extract was obtained through the maceration method using 96% EtOH, and the extract obtained then tested for its inhibitory activity. Based on the results of the observation, all the various traditional Balinese spices extract have inhibition on the growth of *Salmonella sp*. bacteria. Inhibition can be seen from the clear zones that formed around the disk containing traditional Balinese spices extract. In this method, the extract on the disk will diffuse, and the active substances contained in it will inhibit bacterial growth. The wider the clear zone formed shows, the more significant inhibitory activity. In this study, 96% ethanol was used as a negative control to determine whether there is an effect of ethanol on the growth of *Salmonella sp*. bacteria which is also used as a solvent in the extraction process. The mean diameter of negative controls showed there is no inhibitory activity of *Salmonella sp*. bacteria growth with a measured value of 0.00 mm. The same thing was also obtained from the study i*n vitro* inhibitory activity of ethanolic fruit extract from *Averrhoa bilimbi* L. against *Streptococcus pyogenes* bacteria.[5]

In this study, the positive controls used Ciprofloxacin antibiotic discs as work controls. Ciprofloxacin is a broad-spectrum antibiotic that is widely used to treat human and animal diseases, which Ciprofloxacin is a broad-spectrum antibiotic of the fluoroquinolone class. Ciprofloxacin is active against some Gram-positive and many Gram-negative bacteria. Ciprofloxacin has rapidly bactericidal activity and high potency. Relatively long post-antibiotic effect.[5] The mean diameter of the inhibition zone from Ciprofloxacin antibiotic discs was 31.88 mm. These results are according to CLSI (Clinical and Laboratory Standards Institute), where

Ciprofloxacin belongs to the sensitive category. It is indicated that the bacterial isolates used and the procedures carried out in this study are confirmed with testing standards for inhibiting antibacterial substances.[6] This study also shows that Ciprofloxacin is still effective against *Salmonella sp*. bacteria.[7]

The results of this study show that traditional Balinese spices extract (1 mg/ml) were able to inhibit the growth of *Salmonella sp*. Bacteria which was characterized by the formation of clear zones around the discs. This study also shows that different diameter inhibition zones are formed for each traditional Balinese spices extract. It might be caused the differences of spices content and the number of spice compositions (which contain antimicrobial compounds) in traditional Balinese spices. The result of the largest inhibition zone was produced by Basa Gede, which was equal to 19.50 mm, among the other traditional Balinese spices. It might be caused the Basa Gede consists of spice components that are more complete than other spices. Basa Gede contains galangal, ginger, turmeric, shallots, garlic, pepper, coriander, chilli, cumin leaves, bay leaves, Wangen leaves, and lemongrass. The beneficial properties in herbs and spices are due to the presence of phytochemicals. The major phytochemical classes associated with herbs and spices include a diverse array of compounds such as terpenes and terpene derivatives.[8] Other compounds include glycosides, alkaloids like piperine, chavicene, capsaicinoids (pepper fruit), saponins like trigonelline, allyl sulphur, adenosine, (onions and garlic), quercetin (onions), curcumin (turmeric) and isothiocyanates (cruciferous vegetables).[9] Qualitative test results by phytochemical screening indicate the presence of alkaloids, flavonoids, phenols, and tannins contained in the extract. Flavonoid is an active substance which is a class of hesperidin compounds and causes damage to bacterial cell wall permeability, microsomes, and lysosomes as a result of interactions between flavonoids and bacterial DNA.[10]

Phenol is one of the compounds contained in essential oils which has a broad spectrum of bioactivity, owing to the presence of several active ingredients or secondary metabolites, which work through various modes of action.[11] Essential oils contain a variety of volatile molecules such as terpenes, phenolic-derived aromatic and aliphatic components.[12] Phenolic compounds are generally claimed to be bacteriostatic. Moreover, the antimicrobial action of phenols is related to their ability to denature proteins, as well as to produce a loss of membrane integrity resulting in leakage of essential intracellular constituents such as potassium cation, inorganic phosphate, pentoses, nucleotides and nucleosides, and proteins. The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more non-specific interactions with the proteins.[13]

Tannins (commonly referred to as tannic acid) are water-soluble polyphenols that are present in many plant foods. Tannin toxicity for fungi, bacteria and yeasts is reviewed and compared to the toxicity of related lower molecular weight phenols. The dependence of toxicity on tannin structure is examined.[14] The different mechanisms proposed so far to explain tannin antimicrobial activity include inhibition of extracellular microbial enzymes, deprivation of the substrates required for microbial growth or direct action on microbial metabolism through inhibition of oxidative phosphorylation.[15]

The formation of different inhibitory zones due to variations in the types of constituent variations and differences in the number of components and storage conditions.[11] The freshness index of the ingredients of traditional Balinese spices can be seen from the texture of the material, the overall colour of the material, the basic colour of the skin, the colour of the contents of the material, the hardness (firmness) of the material, the content of dissolved sugar (solid soluble content), acidity, and the concentration of ethylene. Harvesting of traditional Balinese spices ingredients which is done earlier or rather late can affect the quality of spices, such as colour, texture, taste and aroma as well as the content of chemical compounds especially those that are harvested earlier or later. This influences changes in chemical and physical properties during

maturation because after harvesting the material still performs metabolic reactions. The optimum temperature for storing materials is 5-10°C. If the temperature is too low, it can cause damage to the material (chilling injury). Other factors that affect the inhibition zone diameter are the incubation temperature, incubation time, tool sterility, contamination, the turbidity of the bacterial suspension, media thickness, and disk disc distance.[16] However, these factors have been controlled, so these factors do not have a significant influence on the results of this study.

This research can be continued by using modifying formulas from traditional Balinese spices, so the concentration of antimicrobial compounds contained becomes higher. This is expected to increase the inhibitory growth of *Salmonella sp.* bacteria.

4. CONCLUSION

Based on the results of the research, it can be concluded that the traditional Balinese spices contain antibacterial compounds such as flavonoids, alkaloids, phenols (3.35 mg/ml) and flavonoids (6.96 mg/ml) and tannin (6.66 mg/ml) with antimicrobial potential categories was intermediate (inhibitory range was 16-20 mm). There are differences in the antimicrobial potential of various types of traditional Balinese spices against the growth of *Salmonella sp.* bacteria in vitro with sig value was 0.037 (<0.05). The result of the largest inhibition zone was produced by Basa Gede, which was equal to 19.50 mm, among the other traditional Balinese spices.

DISCLOSURE STATEMENT

The authors reported no potential conflict of interest.

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SHORT BIOGRAPHY

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Does research in Microbiology. Current research interest include identification of Aspergillus species in Tumeric Jamu. https://scholar.google.co.id/citations?user=YzOjKmkAAAAJ&hl=en