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Euphorbia Milii and Propolis (EMP) Combination tea Maintains Cellular Immunity in Volunteers during the Pandemic of Covid-19 without interfering with the Functions of Liver and Kidney

Ni Made Linawati[1]*, I Wayan Rai Widarta[2], Susy Purnawati[3], I Nyoman Wande[4], Dewa Ayu Agus Sri Laksemi[5], I Gusti Nyoman Sri Wiryawan[1], Indira Vidiari Juhanna[3], I Gusti Ayu Dewi Ratnayanti[1]

[1]*Department of Histology, Faculty of Medicine, Udayana University*

[2]*Department of Food Technology, Faculty of Agriculture Technology, Udayana University*

[3]*Department of Physiology, Faculty of Medicine, Udayana University*

[4]*Department of Clinical Pathology, Faculty of Medicine, Udayana University*

[5]*Department of Parasitology, Faculty of Medicine, Udayana University*

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CORRESPONDING AUTHOR:
Ni Made Linawati,
Email: md_linawati@unud.ac.id

ABSTRACT

Introduction: Euphorbia milii and Propolis (EMP) combination tea have proved to be immunomodulator in animal experimental study. This study aims to show the effect of the EMP tea on immune protection in volunteers during the pandemic of Covid-19 without interfering in the functions of liver and kidney. Methods: The study was performed with 30 volunteers as the subjects. The EMP tea in dose of 0.05 g per kg of body weight was prepared in tea bag and given to the volunteers. The tea was given to the volunteers at tea time once daily for 30 days during the pandemic of Covid-19 in Ketewel regency. The functions of liver (SGOT and SGPT) and kidney (BUN and SC) and cellular immune (WBC, Neutrophils, Lymphocytes and NLR) were tested before and after EMP tea intervention at day 0 and 31.

Results: The level of WBC, Neutrophils, Lymphocytes, SGOT, SGPT, BUN, and SC after EMP tea intervention were normal and there were no significant differences between before and after intervention ($p>0.05$) except for SC level, which showed significant difference before and after ($p<0.05$) although it was still kept in normal range.

Conclusion: The EMP tea protects cellular immunity in volunteers during the pandemic of Covid-19 without interfering with the function of the liver and kidney.

1. Introduction

Natural ingredients as traditional medicines are considerately easy to obtain, cheap, and have minimal or no side effects. Indonesia has a variety of natural ingredients that are trusted and many have proven their potential as drugs. The use of natural ingredients as herbal, standardized drugs, and phytopharmaceuticals must be carried out with preclinical and clinical testing stages[1]. Crown of thorns or *Euphorbia milii* (*E. milii*) flowers containing triterpenoids, saponins, phenolics, flavonoids, and alkaloids have been shown to be immunostimulants in NK cells through increased expression of NKp46[2] and expression of Interleukin-17 in mice infected with *Mycobacterium tuberculosis* (*M.tb*)[3]. Propolis has been shown as an immunomodulator against macrophage cells in TB patients[4]. *Euphorbia milii* and propolis tea is a combination of *E. milii* flower and propolis (from honeycomb). It has an immunomodulatory effect by increasing the secretion of granzyme B and did not cause lung and liver damage in mice infected with *M. tb*[5]. Giving EMP tea did not cause kidney toxicity in mice infected with *M. tb*[6]. The EMP tea at a dose of 40 mg per 100 g body weight taken once time a day (daily) has been shown to prevent atherosclerosis by lowering MMP-8 and total cholesterol but not VEGF- ß in rat with high fat diet[7]. The pandemic of Covid-19 caused an increase in morbidity and mortality worldwide. There were 448,000 cases and 14,800 deaths due to Covid-19 in Indonesia in the year of 2019[8]. The disease caused by the SAR- CoV-2 virus, like other viral diseases, is affected by the host's immune. The infection of SAR-CoV-2 virus caused an excessive host's immune response such as a cytokine storm that causes multi-organ damage[9]. For this reason, it is necessary to maintain the host's immune optimally during pandemic period with natural immunomodulators. This study aims to prove the effect of natural ingredients from *E. milii* flowers and propolis (EMP) tea for maintain cellular immune without interfere with the functions of liver and kidney in volunteers during the pandemic of Covid-19 at Ketewel Bali.

2. Materials And Methods

2.1. *The Design, place, and time of Study*

The study was an experimental study with 30 volunteers as subjects, and they were tested for complete blood count (CBC) for data of the cellular immunity at before and after EMP tea intervention. The subjects consisted of 17 females and 13 males according to inclusion and exclusion criteria i.e physical examination, liver and kidney function within normal range. The study was done at Udayana University (the Laboratory of Food Processing Faculty of Agriculture Technology, the Laboratory of Integrated Biomedical Faculty of Medicine, the Laboratory of Analytic) and the Mantra Medica Clinical Laboratory at Ketewel, Gianyar, Bali. The study was conducted for 6 months in the year 2021.

2.2. *Materials*

Euphorbia milii (*E.milii*) flowers, honeycomb or tala, tea paper bag, reagen from Erba for SGOT (Serum Glutamic Oxaloacetic Transaminase), SGPT (Serum Glutamic Piruvic Transaminase), BUN (Blood Urea Nitrogen), SC (Serum Creatinine) and reagents from Sysmex (cell pack, stromatolites and cell clean) for CBC (Complete Blood Count).

2.3. *Instrumentations*

Blender (Philips series 5000) to cut E.milii flower and honeycomb into a small pieces; Dryer Oven (Memmert UN55) to dry *E.milii* flower and the honey comb; impulse heat sealer to seal tea bag; Hematology analyzer (Sysmex XP 100) to process complete blood count such as WBC, neutrophils, lymphocytes; Chemical analyzer from Erba XL 100 to process the blood chemistry test as SGOT, SGPT, BUN and SC; disposable syringe 5 ml (BD), blood collecting tube, centrifuge tube, centrifuge (WINA instruments; type 507; rotor 12 tube; Centrifuge Hi Speed Electromotor to separate serum from the blood), spectrophotometer (UV-1800; Shimadzu UV Spectrophotometer serial no A114549 O7235) to examine the phytochemical content of EMP tea.

2.4. The Preparation of EMP tea

E.milli flowers are picked from a flower plantation in Ketewel, Gianyar, Bali. Approximately 1000 grams are cleaned and put in a drying oven at a temperature of 40°C for 24 hours. Drying flower then was blended and then 400 grams of E.*milii* powder was produced. Propolis were obtained from the honeycomb which was found on a honey bee farm at Plaga, Badung, Bali. Approximately 1200 g of honeycomb or tala which was been empty and cleaned, then was cut into small pieces before dried in a drying oven at a temperature of 40°C for 48 hours. Approximately 600 g of honeycomb powder were produced and ready to be mixed with *E.milii* powder. The EMP tea from *E.milii* flower and propolis (tala) was made by mixturing powder of 400 g *E.milii* and 600 g tala. After the two ingredients are mixed well, it is ready to be packed into the tea bag. The doses 0.05 g per kg of body weight daily were prepared in tea bag and then brewed with 100 ml hot water and ready to consume after cooling.

2.4. *Phytochemical and nutritional examination of EMP tea brew*

The EMP tea brew was examined for its phytochemical and nutritional in accordance with standard procedure content by laboratory staff of the Analytical laboratory at Udayana University.

2.4.1. Total Phenolic

Determination of total phenolic by Folin–Ciocalteau method [10]. Folin-Ciocalteu reagent was diluted with water at a ratio of 1:9 (v/v). To in 1.25 ml of reagent, 50 µl of sample was added. After that, it was incubated for 2 minutes at room temperature, then added 1 ml of sodium carbonate (75 g/l). The mixture was incubated for 15 minutes at 50°C and quickly cooled in container filled with ice water. In 15 minutes the absorbance was read at wavelength 760 nm. Reading results were compared with the standard curve which was constructed using gallic acid at concentrations of 0, 25, 50, 75, 100, 150, 200 ppm. The standard curve equation is:
$y=0.0023x+0.0057$, $R^2=0.9991$.

2.4.2. Total Flavonoid

A total of 1 ml of sample was mixed with 4 ml distilled water and 0.3 ml of NaNO2 solution (5%). After 5 minutes, 0.3 ml of AlCl3 solution (10%) was added, then vortexed and left for 6 minutes. Then 2 ml of NaOH solution (1 M) was added and 2.4 ml of distilled water. The absorbance of the solution was measured at a wavelength of 510 nm. The quercetin standard curve was prepared using concentrations 0, 2, 4, 6, 8, 10, and 12 mg/ml. The standard curve equation is: $y=0.0011x+0.0017$, $R^2=0.9997$.

Concentration of flavonoids in the test sample is calculated from the calibration standard curve and stated as quercetin equivalents in mg/g sample[11].

2.4.3. IC_{50} (Antioxidant activity)

A total of 3 ml of DPPH (0.004% w/v in methanol) was dissolved in 100 µl of avocado leaf extract (1% concentration) in a test tube. The solution was incubated for 30 minutes in the dark at room temperature. The absorbance is read at a wavelength of 517 nm against control using methanol as blank. The percentage of the ability to capture free radicals (antioxidant activity) is calculated by equation:

$$\% = \frac{Ao - As}{Ao}\ x100$$

Where Ao: the control absorbance and As the sample absorbance.

Next the calculation results are entered into the regression equation Y = aX + b.

Where Y: % inhibition value (activity antioxidant) and X: the extract concentration (100-2000 mg/L). IC_{50} value is derived from the calculation 50% inhibition[12]

2.4.4. Vitamin C

Vitamin C or ascorbic acid (AA) determination

Table 1: The general characteristics of volunteers

Characteristics	Volunteers
Age (years old)	**Persons (%)**
25-35	11 (36.67)
36-46	19 (63.33)
Sex	**Persons (%)**
Male	13 (43%)
Female	17 (57%)
Physical Examination (general sign)	**30 persons (100%)**
Blood pressure (mmHg)	110/70 – 125/85
Resting heart rate (beats per minute)	70 – 85
Respiratory rate (breaths per minute)	16 – 20
Body Mass Indexes /BMI (%)	19.5 – 22

was performed by simple redox titration methods using 2.6-Dichlorophenolindophenol (DCPIP). A solution of DCPIP was calibrated by 2 mL standard solution of AA, which contained 1.08 mg. Based on the obtained data each mL of DCPIP solution was equivalent to 0.093 mg of AA. Then the samples of EMP brew were titrated by calibrated titrant. After titrations, results were expressed as mean ± confidence limits at 95% confidence level[13]

2.4.5. β-Carotene

The concentration of β-carotene was determined using U-HPLC. The U-HPLC system (LaChromUltra L-2000 U Series; Hitachi-High Technologies Corp., Hitachinaka, Japan) was equipped with an eluant reservoir, a U-HPLC pump (Model L-2200U), an autoinjection system of 5 µL injection at a fixed volume. LaChromUltra C18 (2 µm, 2 mm i.d.×50 mm L, Hitachi-High Technologies Corp.) was used as an analytical column. Mobile phase was ethylacetate: acetonitrile: acetic acid (30:68:2, v/v/v) with 0.22 mM BHT and flow rate was 0.2 mL/min. Detector was UV detector (Model L-2400U; Hitachi-High Technologies Corp.) set at the wavelength of 450 nm. Calibration graph for U-HPLC was based on peak area and prepared by injecting 5 µL of 0.5, 1.0, 5.0, and 25.0 µg/ mL solutions prepared by the dilution of β-carotene stock solutions with a mobile phase[14]. The standard curve equation is: y=174.33x-18.35, R^2=0.999

2.4.6. Mineral composition

Mineral composition of the samples was determined according to methods recommended by Association of Official Analytical Chemists [15]. The samples were incinerated in the oven at a temperature of 550°C for 3 hours. The samples of EMP tea was digested using a mixture of concentrated Nitric (HNO_3), perchloric ($HClO_4$) and sulphuric ($H2SO_4$) acids in the ratio 9:2:1 (v/v) respectively. Copper (Cu), iron (Fe), zinc (Zn), sodium (Na), calcium (Ca) and magnesium (Mg), manganese (Mn), and lead (Pb) were determined by Atomic Absorption Spectrophotometer (AAS) (PerkinElmer Analyst 700, England). Details are explained in Adjatin *et al.* (2013)[16]

2.4.7. Proximate analysis

The sample was analysed for moisture, crude protein, and ash content. Crude protein was determined by using the Kjeldahl method. The moisture was determined according to the procedure of Association of Official Analytical Chemist (AOAC, 1990).

The percentage was calculated based on the dry weigh. Ash was determined after incineration in a muffle furnace[16]

2..4.8. Sugar

Total sugars in the samples of EMP tea brew were determined according to methods recommended by AOAC 968.28-1969 (2000)[17]. And the result of total sugars were expressed in percentages.

All experiment were carried out in triplicate and the results were represented by mean and standard deviation. The phytochemical results are presented in the Table 2.

2.5. *The intervention of EMP tea for the healthy volunteers*

The volunteers were given EMP tea in dose of 0.05 g per kg of body weight daily for 30 days. The EMP tea was given during tea time, between 10 am – 12 am. During the EMP tea consumed, the research has only standardized the diet only for the prohibition of vitamin and other nutritional supplements, whereas the daily meal was according to the subject's usual diet.

2.6. *The liver functions (SGOT and SGPT) and kidney functions (BUN and SC) tests at before and after EMP tea intervention*

The volunteers with normal clinical signs were approved and informed and provided consent. The diet control during the test is according to the protocol of variable of interest, i.e fasting for 8 hours prior testing (only drinking water were allowed). Approximately 2 ml of blood were collected in a tube without anticoagulant from mediana cubiti vein with a standard procedure. The blood sample was then processed according to the procedure of an Erba XL 100 chemistry analyzer with Erba reagen for SGOT, SGPT, BUN, and SC. The test was done before and after EMP tea intervention.

2.7. *Determination of cellular immunity from complete blood count (WBC, lymphocytes, neutrophils and NLR)*

Blood samples from volunteers were collected by laboratory staff according to standard procedures. Two ml of blood was taken from Mediana Cubiti vein and were put into an anticoagulant tube for a further process according to standard procedure for complete blood count examination using Sysmex XP100 hematology analyzer with reagent cell pack, stromatolites, and cell clean from Sysmex.

2.8. *Statistical Analysis*

The data of immune cells (WBC, lymphocytes, neutrophils and NLR), liver functions (SGOT and SGPT) and kidney functions (BUN, and SC) on before and after EMP tea intervention were tabulated, described, and analyzed using SPSS version 24.

3. **Results and Discussion**

3.1. *Results*

The general characteristic of volunteers are shown in Table 1. There were 30 healthy volunteers who qualified, consisting of 43% male and 57% female. The mean age was 36.67% between 25 - 35 years old and 63.33% between 36 – 45 years old. All volunteers (100%) have normal body mass index (BMI), blood pressure, heart rate, temperature, and respiratory rate. They did not have any complaints such as fever, coughs and colds.

The feature of cellular immune, liver and kidney function, of the volunteers is described in Figure 1. The features of immune cells in the volunteers could be seen from CBC i.e the number of white blood cells (WBCs), neutrophils, lymphocyte and NLR in before and after EMP tea intervention. The mean number of WBC before and after intervention were still in the normal range (ref. value in male: 4.1-11x 10^3/µl; female 4.1-11x 10^3/µl). The same condition happens in the mean of the neutrophil percentage before and after EMP tea intervention was also in the normal limit (ref. value: in male: 50-70%; female: 20-60%). Similarly with the mean of lymphocytes percentages in before and after EMP tea intervention were also in the normal limit (ref. value in male: 13-40%; in female: 30-64%). From the results, there were no

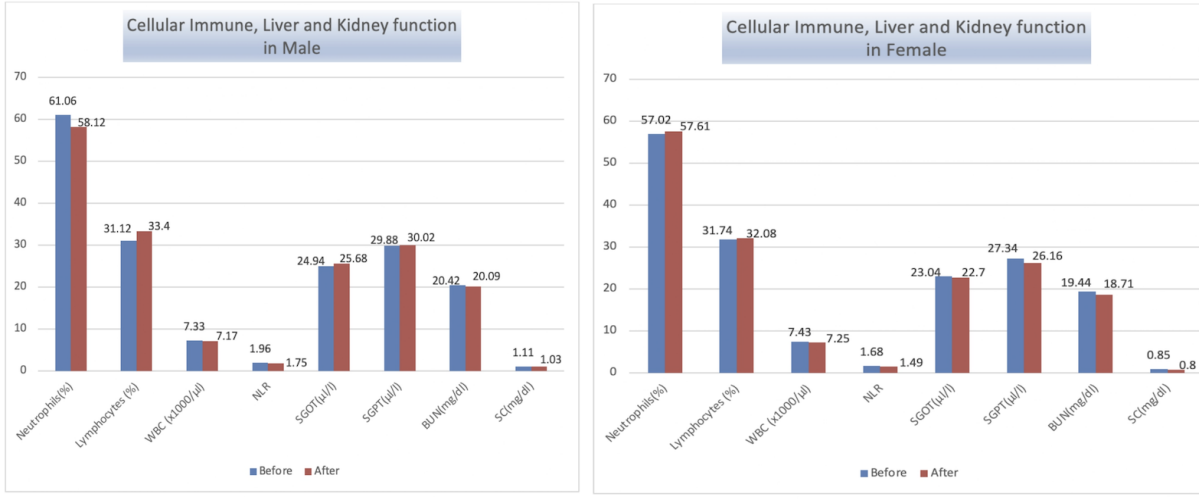

Figure 1. *The Feature of Cellular immune, Liver and Kidney functions in Volunteers*

significant difference between before and after EMP tea intervention in the number of WBC, neutrophil percentage and lymphocytes percentage of the volunteers. There were also no significant difference in the neutrophils-lymphocytes ratio (NLR) between before and after EMP tea intervention. It seems that giving EMP tea is able to replace the role of multivitamins or supplements that might be consumed before the tea intervention in maintaining cellular immunity of volunteers. In male, the mean level of SGOT and SGPT before and after EMP tea intervention was still in the normal range (reference value for SGOT: 10-40 µl/l; SGPT: 10-55 µl/l). Similarly in female, the mean level of SGOT and SGPT before and after the administration of EMP tea was also in the normal range (reference value for SGOT: 9–25 µl/l; SGPT: 7 – 30 µl/l). The result also showed no significant difference ($P > 0.05$) between before and after intervention values of SGOT and SGPT both in male ($P = 0.822$) and female ($P = 0.863$). The mean value of kidney functions (BUN and SC) in male and female in before and after EMP tea intervention were shown in normal limit. In which the normal value in male (BUN: 8-25 mg/dl; SC: 0.6-1.5 mg/dl) and female (BUN: 8-25 mg/dl; SC: 0.5-0.9 mg/dl). The result showed there are no significant difference ($P > 0.05$) between before and after EMP tea intervention in BUN level but it was significant difference in SC level ($P = 0.030$). It seems, EMP tea intervention did not interfere with liver and kidney function in volunteers so it was safe for consumption.

The results of the phytochemical and nutritional examination of EMP tea are described in Table 2. The EMP tea brew contains polyphenols, flavonoids, antioxidants, vitamin C, beta carotene, glucose, protein, Fe, Zn, Ca, Mg, Mn, Na, water content, ash content, Cu, and Pb. The EMP tea brew has good content and did not contain heavy metals in concentrations that are harmful to the volunteers.

3.2. Discussion

This study is a continuation from previous EMP experimental study in mice and rats. Previous studies concern the effect of *Euphorbia Milli* tea and its combination with propolis on a number of glomeruli in *Mycobacterium tuberculosis* infected mice. It was found that the Euphorbia milii tea and its combination with Propolis does not affect histological change on the total number of glomerulus in mice infected with *M. Tb*[7] so EMP tea does not impair renal fuction. Other studies found that *Euphorbia milii* and propolis combination tea (EMP) did not cause liver tox-

Table 2: The Phytochemical and nutritional content of EMP tea brew

Parameter	Unit	Results
Polyphenols	mg/mL GAE	55 ± 0.05
Flavonoids	mg/mL QE	1.32 ± 0.01
IC_{50}	mg/mL	1.65 ± 0.02
Vitamin C	mg/mL	13.2 ± 0.05
β-Carotene	mg/mL	0.979
Water content	%	99.895 ± 0.02
Ash content	%	0.003 ± 0.0001
Protein	%	0.077 ± 0.002
Sugar	%	0.08 ± 0.002
PH	A-	6.2 ± 0.01
Iron (Fe)	ppm	7.44
Zinc (Zn)	ppm	16.6
Calcium (Ca)	ppm	24.6
Magnesium (Mg)	ppm	5.49
Manganese (Mn)	ppm	0.083
Sodium (Na)	ppm	6.77
Copper (Cu)	ppm	2.419
Lead (Pb)	ppm	0.204

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icity and lung damage while affecting granzyme secretion in *Mycobacterium tuberculosis infected mice*[6]. It has also been reported that EMP combination tea increases the diameter of lymphoid nodule (white pulp) in the rat spleen[18]. Moreover, *Euphorbia milii* flower and propolis (EMP) combination tea prevents atherosclerosis through decreasing MMP-8 and total cholesterol level but not VEGF-β in rat with high-fat diet (HFD)[7]. In connection with immunomodulator potency and lack of liver and renal toxicity in animal experimental studies above, the present follow up study aims to prove the protective effects of EMP tea in volunteers during the pandemic of Covid-19, without interfering with the functions of the liver and kidney. The results of the liver functions (SGOT, SGPT) and renal functions (BUN, SC) in before and after the EMP tea administration showed normal limits, although there was a significant difference ($P < 0.05$) in the level of serum creatinine (SC) between before and after EMP tea intervention both in male ($P = 0.030$) and female ($P = 0.044$). After 30-day administration of EMP tea there was a decrease in SC level. This condition indicated as a favorable condition, which is beneficial and may be partly contributed to EMP tea administration. The presence of phytochemicals and nutrients in the EMP tea brew i.e polyphenol, may have a protective effect, as supported by Fernandes and Costa.[19]. The EMP tea also contains flavonoids which have been shown to reduce kidney damage[20]. Iron (Fe) in EMP tea has also a protective effect on kidney function[21] as well as beta carotene[22]. Zinc in EMP tea also supports kidney function. Zinc deficiency has been reported to be a risk factor for chronic kidney disease progression[23]. The infection by Sar-CoV-2 caused the interaction of virulence agent with the host immunity.

The main immune response to viral infections was innate and acquired cellular immune responses[24]. In general, cellular immune responses to viral infections virus are mediated by white blood cells (WBC) which circulate throughout the body through blood vessels and migrate to lymph nodes via high endothelial venules (HEV). The regulation of the immune system can be assisted by using immunomodulator substances from natural resources i.e EMP tea[7]. The excessive immune response to SAR-CoV-2 infection has been reported to induce a cytokine storm due to excessive pro-inflammatory cytokines that damage tissues and then cause various multi-organ failure (MOF), which ends in death[9]. The mean of neutrophil percentage before and after the EMP tea intervention was in normal limit. Similarly was the mean lymphocytes percentage. Neutrophil- lymphocyte ratio (NLR) values before and after intervention were also within normal limits. but there was a decrease in NLR although the decrease was not statistically significant. The decline in NLR values during the pandemic of Covid-19 indicates a positive situation, where high NLR values are often associated with an inflammatory state[25]. The flavonoid have previously been studied to have an effect on NLR values. Flavonoid in Dayak onion bulb extract increase the production of IL-2 which is involved in the activation and proliferation of lymphocytes. Flavonoids also have an anti-inflammatory potential through their ability to modulate the expression of pro-inflammatory genes[26,27]. The other compounds and minerals in EMP tea such as zinc (Zn) and iron (Fe) reduce NLR. This was supported by research from Zhou et al[28]. Iron deficiency causes immune system dysfunction and affects the proliferation of T lymphocytes. Other studies reported that zinc was a micronutrient with its anti-SARS-CoV-2 viral effect and it could modulate the immune response. Deficiency of selenium, iron, and zinc is associated with the abnormal regulation of neutrophils and lymphocytes in Covid-19 patients and influences NLR and severity of the disease[29,30,31]. The present study shows, that EMP tea maintains optimal cellular immunity (WBC, neutrophils, leukocytes) as before tea intervention. The volunteers consumed immunomodulatory supplements or other multivitamins freely before tea intervention, while during EMP intervention for 30 days they were free from any supplement. This indicated that EMP tea has an immunomodulatory effect so that cellular immunity conditions remain optimal even during the Covid-19 pandemic and its also safe for the liver and kidney.

4. Conclusion

The administration of EMP tea with a dosage of 0.05 g per kg of body weight daily for 30 days maintains cellular immunity of volunteers without interfering with the functions of the liver and kidney during the pandemic of Covid-19 in Ketewel, Bali.

Ethics Committee

This research has received ethical approval from the research ethics committee of the faculty of Medicine Udayana University according to number: 2607/UN14.2.2.VII.14/LT/2021.

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Conflict of interest

The Authors declare no conflict of interest.

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