

Study of immunomodulatory activity of electroacupuncture technology and *Hylocereus undatus* fruit extract in rats

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ABSTRACT

The purpose of the present study was to describe the mechanism of electroacupuncture technology and standardized fruit extract of *Hylocereus undatus* as an immunomodulator in rats. It was an experimental study with a post-test only control group design. Samples consisted of 30 to male white rats aged 5-6 months and weighed 250 grams, which were divided into five treatment groups and 6 replications in every group. An electroacupuncture technology was applied the acupuncture points of Hegu (Li 4), Zu San Li (ST.36), and Sanyinjiao (L.6) in order to enhance cellular and humoral immune responses. In vitro tests of immunomodulatory activity were conducted in 4 stages: isolation of macrophages, isolation of lymphocytes and macrophage activity test, blast transformation test with MTT assay, standardization extract of *Hylocereus undatus* fruit with the TLC method and identification of *Hylocereus undatus* conducted at the Purwodadi Botanical Garden, Pasuruan, Indonesia. The data was analyzed by using ANOVA. We showed that *Hylocereus undatus* fruit extract combined with the electroacupuncture technology was capable of enhancing and activating phagocytic activity of macrophages in the cellular immune responses and activating B lymphocytes in the humoral immune responses to the antigen of canine parvovirus (CPV) and increasing the levels of lymphocytes, monocytes, erythrocytes, and hemoglobin of the blood feature of the rats infected with CPV antigen.

Key words : Canine parvovirus, Electroacupuncture, *Hylocereus undatus*, immunomodulator.

Introduction

CPV infection is among the infectious diseases caused by CPV. The disease is often found in dogs

of various breeds and ages, in various seasons in Indonesia. The clinical signs of CPV infection include vomiting and defecation, accompanied by blood and distinctive smell. Cases of CPV infection

are frequently reported to hit Asian regions, particularly Indonesia and China. Pets highly susceptible to the illness are dogs. CPV infection is most commonly found in unvaccinated dogs. It often causes hemorrhagic diarrhea, if occurs persistently, will lead to a highly dehydrated condition in the infected dog, causing death. The average mortality of dogs due to CPV infection is very high, reaching 91%. If medication is more immediately performed, the average dog mortality can be reduced up to 48% (Tabor, 2011; Zhao *et al.*, 2013).

Electroacupuncture is the stimulation of acupuncture points administered by pricking a needle electrified with a frequency of 2 Hz and a voltage of 3-5 volts (Rejeki *et al.*, 2016). Electroacupuncture is often conducted in humans and animals aimed at enhancing the body's cellular and humoral immune responses to a specific disease by stimulating specific points, including Hegu (Li.4), Zu San Li (ST.36), and Sanyinjiao (L.6). Electroacupuncture technology on pets or livestock is applied only for the purpose of eliminating pain, nervous system disorders, increased milk production, weight gain, and improved animal performance. Electroacupuncture technology to treat infectious diseases are rarely applied to animals (Zhang *et al.*, 2014; Zhou *et al.*, 2019).

Medicinal plants were used in many developing countries with natural diversity resources, including Indonesia (Fitratullah *et al.*, 2019; Ansori *et al.*, 2019; Ansori *et al.*, 2020; Fadholly *et al.*, 2019; Fadholly *et al.*, 2020; Purnamasari *et al.*, 2019; Sukardiman and Ervina, 2020; Rahmadhani *et al.*, 2020). *Hylocereusundatus* is one the herbal plants found in Indonesia which is very useful and has many active ingredients, including minerals, vitamins, alkaloids, phenols (tannins) and flavonoids containing anthocyanins and betacyanins (Pribadi *et al.*, 2014; Suh *et al.*, 2014; Hapsari *et al.*, 2017; Som *et al.*, 2019; Budi *et al.*, 2019). *Hylocereusundatus* contain antioxidants and can be used for the treatment of such various diseases as gout, diabetes, arthritis, inflammation, and others (Suh *et al.*, 2014; Som *et al.*, 2019; Wahdaningsih *et al.*, 2020). Thus, a treatment strategy for CPV infection using a combination of the electroacupuncture technique at specific points that stimulate cellular and humoral immune responses in animals and the administration of *Hylocereusundatus* containing alkaloids, phenols (tannins), vitamins, and minerals is expected to stimulate T and B lymphocytes and accelerate cellu-

lar and humoral immune responses in the body, thereby accelerating the activation of phagocytic cells, macrophages, and IgG in killing and eliminating CPV.

Materials and Methods

Materials

A total of 30 male white rats were obtained from EXO GRIMM Farm, Surabaya, Indonesia. Certified laboratory animals and animal ethical clearance were obtained from the Faculty of Dentistry, Universitas Airlangga, Surabaya, Indonesia. The present study used cages, feed, microscope, absolute ethanol solution, and water injection. A total of three kg of *Hylocereusundatus* fruits were derived from Batu, Indonesia. Additionally, this study also used medicine bottles, drug etiquettes, 1 mL syringes, FeCl₃ reagent cotton, sterile distilled water, water baths, microscope, shearer, scalpels, RPMI solution, chloroform, Giemsa dye solution, heparin-containing venoject tubes, and filter papers. CPV antigen of three vials was purchased from the veterinary medicine manufacturer Zoetis. The present study also used extraction tubes, surgical equipment, electroacupuncture instruments, waterbaths, thin-layer chromatography (TLC), and amicroscope.

Study

The study was conducted from June 2015 to August 2016, at the Laboratory of Pharmacology and Pharmacy, Universitas Wijaya Kusuma, Faculty of Pharmacy, Universitas Airlangga, and Laboratory of Animal, Faculty of Veterinary Medicine, Universitas Wijaya Kusuma, Surabaya, Indonesia.

Determination of *Hylocereusundatus*

The roots, stems, leaves, fruits, barks, and seeds of newly picked fresh *Hylocereusundatus* fruits were delivered to the Purwodadi Botanical Garden, Pasuruan, Indonesia in order to determine the species of *Hylocereusundatus* with medicinal efficacy (Fadholly *et al.*, 2019; Fadholly *et al.*, 2020).

Preparation of *Hylocereusundatus* Fruit Extract

A total of 2000 g of *Hylocereusundatus* fruits flesh powder was put into an extraction flask and soaked in ethanol at a ratio of powder and solvent of 1:3. The solution was shaken as much as possible and is filtered with a filter paper after 48 hours. Subse-

quently, the ethanol extract of *Hylocereusundatus* fruits was evaporated using a rotary vacuum evaporator at a maximum temperature of 40°C to obtain viscous extracts (Pribadi *et al.*, 2014; Wahdaningsih *et al.*, 2020).

Standardization of *Hylocereusundatus* Fruit Extract

Standardization of *Hylocereusundatus* fruit extracts was conducted qualitatively by dripping the reagent Fe₂Cl₃ to identify the derivative chemical compounds of alkaloids, flavonoids, phenols (tannins), triterpenoids, saponins, and etc. In order to more thoroughly standardize the active chemical constituents most abundantly found in the *Hylocereusundatus* fruits extract using thin-layer chromatography (TLC).

Preparation of *Hylocereusundatus* Fruit Extract Injection

Standardized viscous *Hylocereusundatus* fruit extracts were dried to dry and then weighed at the dosage of 10 mg, 15 mg, and 20 mg. Subsequently, 50 mL of sterile water injection solution as vehicle was added to make *Hylocereusundatus* fruit extract injection drug preparation.

Treatment of Laboratory Animals

A total of 30 white male rats was divided into five groups and six replications. Those groups were the control group of healthy rats without CPV antigen induction and treatment (P0), the group of rats induced with CPV antigen and treated with electroacupuncture twice a day (P1), the group of rats induced with CPV antigen and treated with electroacupuncture twice a day in combination with administration of 10 mg of *Hylocereus undatus* fruit

extract drug injection (P2), the group of rats induced with CPV antigen and treated with electroacupuncture twice a day in combination with administration of 15 mg of *Hylocereusundatus* fruit extract drug injection (P3) the group of rats induced with CPV antigen and treated with electroacupuncture twice a day in combination with administration of 20 mg of *Hylocereusundatus* fruit extract drug injection (P4). The rats were treated for 15 days and subjected to blood sampling. Cardiac blood of 1-2 mL was collected for blood examination in order to determine the features of lymphocytes, monocytes, erythrocytes (RBC) and hemoglobin (HGB) on days 7 and 15. Peritoneal fluids of the rats were examined and collected for isolation of macrophages, isolation of lymphocytes, phagocytic activity testing of macrophages, and blast transformation test by using the MTT assay. These stages of testing were carried out to test herbal medicinal activity as an immunomodulation of CPV *in vitro*. Peritoneal fluids were collected on days 7 and 15. Phagocytic cell activity value was determined by using the following formula: Total % activity = % macrophage activity + % PMN cell activity.

Results and Discussion

Results of active compound standardization of the *Hylocereusundatus* fruit extract are shown in Table 1, the average of lymphocyte count shown at Table 2, the average monocyte count shown in Table 3, the average erythrocyte count at Table 4, and the average HGB count shown at Table 5.

The use of varying concentrations of *Hylocereus undatus* fruit extracts and electroacupuncture tech-

Table 1. Results of active compound standardization of the *Hylocereusundatus* fruit extracts.

No	Active Compound	Reagent Dripping	TLC Equipment
1	Alkaloids	Bright orange	Faint orange
2	Phenols (tannins)	Turbid black	Clear black
3	Flavonoids	Dark red	Faint blackish brown
4	Saponins	Persistently frothy	Little froth
5	Triterpenoids	Purplish black	Purplish red

Table 2. Average lymphocytecount.

Day	P0	P1	P2	P3	P4
0	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
7	9.85 ± 0.11	3.16 ± 0.06	7.44 ± 0.51	8.86 ± 0.48	9.24 ± 0.16
15	10.61 ± 0.70	6.00 ± 0.51	7.50 ± 0.08	9.88 ± 0.29	11.23 ± 0.56

nique served to determine whether or not there was an association between increased concentrations of the administered test extracts and the induced phagocytic activity of macrophages, PMN cells, and lymphocytes. Additionally, it served to determine the types of and at what concentrations the *Hylocereusundatus* fruit extracts tested would work effectively as an immunomodulation of CPV. Results of examination of the phagocytic activity of macrophages, PMN cells and lymphocytes by means of Giemsa staining on days 7 and 15 of treatment for control group (P0) showed that at normal counts, no activation of macrophages, PMN cells and lymphocytes was observed. The group of rats treated with electroacupuncture technique twice a week (P1) showed decreased normal counts and no activation of macrophages, PMN cells and lymphocytes was observed. Phagocytosis testing of macrophages and polymorph nuclear cells (PMN) against CPV for the healthy control compared to the groups of diseased rats only treated with electroacupuncture technology (P1) and the group of diseased rats only treated with electroacupuncture technology (P2) and administered with *Hylocereusundatus* fruit extracts at doses of 10 mg (P2), 15mg (P3), and 20 mg (P4) showed highly significant differences, in which P4 was the most effective immunomodulation of CPV compared to P3, P2, P1, and P0.

The group of rats treated with

electroacupuncture technique twice a week and administered with *Hylocereusundatus* fruit extracts at a dose of 10 mg (P2) showed an increase in macrophage, PMN cell and lymphocyte counts relative to the normal counts and a 20% increase in the activation of macrophages and PMN cells. The group of rats treated with electroacupuncture technique twice a week and administered with *Hylocereusundatus* fruit extracts at a dose of 15 mg (P3) showed an increase in macrophage, PMN cell and lymphocyte counts relative to the normal counts and a 60% increase in the activation of macrophages and PMN cells. The group of rats treated with electroacupuncture technique twice a week and administered with *Hylocereusundatus* fruit extracts at a dose of 20 mg (P4) showed an increase in macrophage, PMN cell and lymphocyte counts relative to the normal counts and a 80% increase in the activation of macrophages and PMN cells. Modified

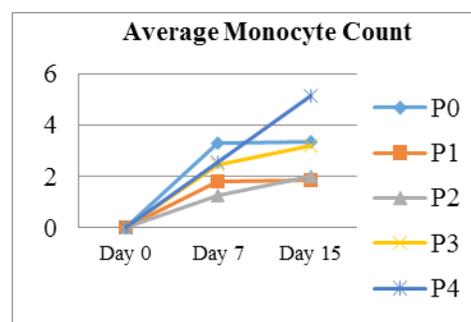


Table 3. Average monocytecount.

Day	P0	P1	P2	P3	P4
0	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
7	3.28±0.07	1.79±0.07	1.27±0.05	2.47±0.09	2.57±0.23
15	3.33±0.34	1.84±0.19	2.02±0.42	3.21±0.28	5.15±0.09

Table 4. Average erythrocytecount.

Day	P0	P1	P2	P3
0	0±0.00	0±0.00	0±0.00	0±0.00
7	7.69±0.12	7.65±0.08	7.31±0.58	8.45±0.05
15	7.79±0.17	7.69±0.18	7.91±0.29	8.52±0.17

Table 5. Average HGB count.

Day	P0	P1	P2	P3	P4
0	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
7	15.00±0.54	12.40±1.23	12.62±0.18	13.63±0.26	17.37±0.16
15	15.88±0.81	13.04±0.41	15.48±0.26	16.08±0.49	17.65±0.14

electroacupuncture technology and *Hylocereusundatus* fruit extract at a dose of 20 mg (P4) provided a significantly different result relative to the average counts of lymphocytes, monocytes, RBC, and HGB in comparison with P3, P2, P1, and P0.

The increase in the average count of lymphocytes, monocytes, RBC, and HGB on days 7 and 15 was due to the course of the disease that turned from being initially acute into chronic and lymphocytes and monocytes which are immune cells being activated to phagocytize CPV. The increase in the average count of lymphocytes and monocytes was due to presence of such active compounds as alkaloids, tannins, flavonoids and triterpenoids as well as minerals in the *Hylocereusundatus* fruit extracts combined with stimulation at three acupuncture points of Hegu (Li 4), Zu San Li (ST.36), and Sanyinjiao (L.6), representing the main points that activate the cellular and humoral immune responses. The increasing average count of RBC and HGB was due to the presence of vitamins and minerals in the *Hylocereusundatus* fruit extracts combined with stimulation at three acupuncture points of Hegu (Li 4), Zu San Li (ST.36) and Sanyinjiao (L.6) that served to stimulate the body's humoral immune responses to produce antibodies (immunoglobulin) that are highly effective against the virus and enclose the cells, leading the cells to become weak and eventually be able to be eliminated out of the body. Increased RBC and hemoglobin usually occur along with an increasingly enhanced immune system (antibody) to resist the disease-causing infectious agents.

Active compounds, such as alkaloids, phenols (tannins), flavonoids and triterpenoids in *Hylocereusundatus* fruit extracts, are those compounds very effectively used as an anti-virus. Alkaloids are a compound that is highly effective in inhibiting viral metabolism of carbohydrates, leading the virus to be unable to store food reserves and produce energy for their survival (Wahdaningsih *et al.*, 2020). Phenols (tannins) are a compound that highly effectively destroys or lyses the cell walls and the viral envelope that leads the virus to become weak and inactive. Flavonoids are a compound that serves to inhibit the viral synthesis of proteins in the body by inhibiting the synthesis of essential amino acids which are an important substance for the formation of proteins. In the event that the viral synthesis of proteins is inhibited, the

viral growth and replication to proliferate and become viral pathogens can also be inhibited. Furthermore, it leads the virus to be more easily phagocytized by immune cells and ultimately capable to be eliminated out of the body. Triterpenoids are a compound that serves to inhibit steroidal compounds in the viral body constituents. Viruses would be highly pathogenic when they are able to replicate and grow rapidly toward different locations of the body. In the event that the steroid compounds in the viral body constituents are inhibited, the virus would be unable to replicate and proliferate rapidly. The virus can eventually become less pathogenic and even weak and is finally easier to be phagocytized by the immune cells such as macrophages or PMN cells (Tabor, 2011).

Treatment with the modified electroacupuncture technology twice a week and *Hylocereusundatus* fruit extracts constitutes an appropriate method to use as an immunomodulation to resist CPV infection. This combined method was able to activate macrophages, PMN cells and lymphocytes, which are the components of the cellular immune responses, and to stimulate faster antibody production, which is the component of the body's humoral immune responses. When the cellular and humoral immune responses are more rapidly and increasingly activated simultaneously, CPV would be more easily phagocytized by the immune cells and weakened/inactivated by antibodies and they would be more rapidly eliminated out of the CPV. When the viral cells have been eliminated, the CPV-infected patient can be cured and the mortality rate of the infected patients can be reduced (Zhou *et al.*, 2019).

Conclusion

In conclusion, we conclude that the highest increase in the activity of macrophages, PMN cells, and lymphocytes is shown by group P4 (80%). The increase in the average count of lymphocytes, monocytes, RBC, and HGB shows a highly significant difference for group P4. Treatment with modified electroacupuncture twice a week combined with standardized fruit extract of *Hylocereus undatus* at a dose of 20 mg (P4) is the best immunomodulator of CPV.

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