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Abstract

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magnification, then the mean value is taken. The mean value of the number of immunoreactive cells is included as data. Data analysis by one way ANOVA and Duncan test. **Results:** The treatment group showed that IFN–γ expression in mice tuberculosis was significantly increased ($p < 0.05$) of EMDU. **Conclusions:** Administration of EMDU increase of IFN–γ expression in mice tuberculosis

Keywords: Graptophyllum pictum (L) Griff, immune, tuberculosis.

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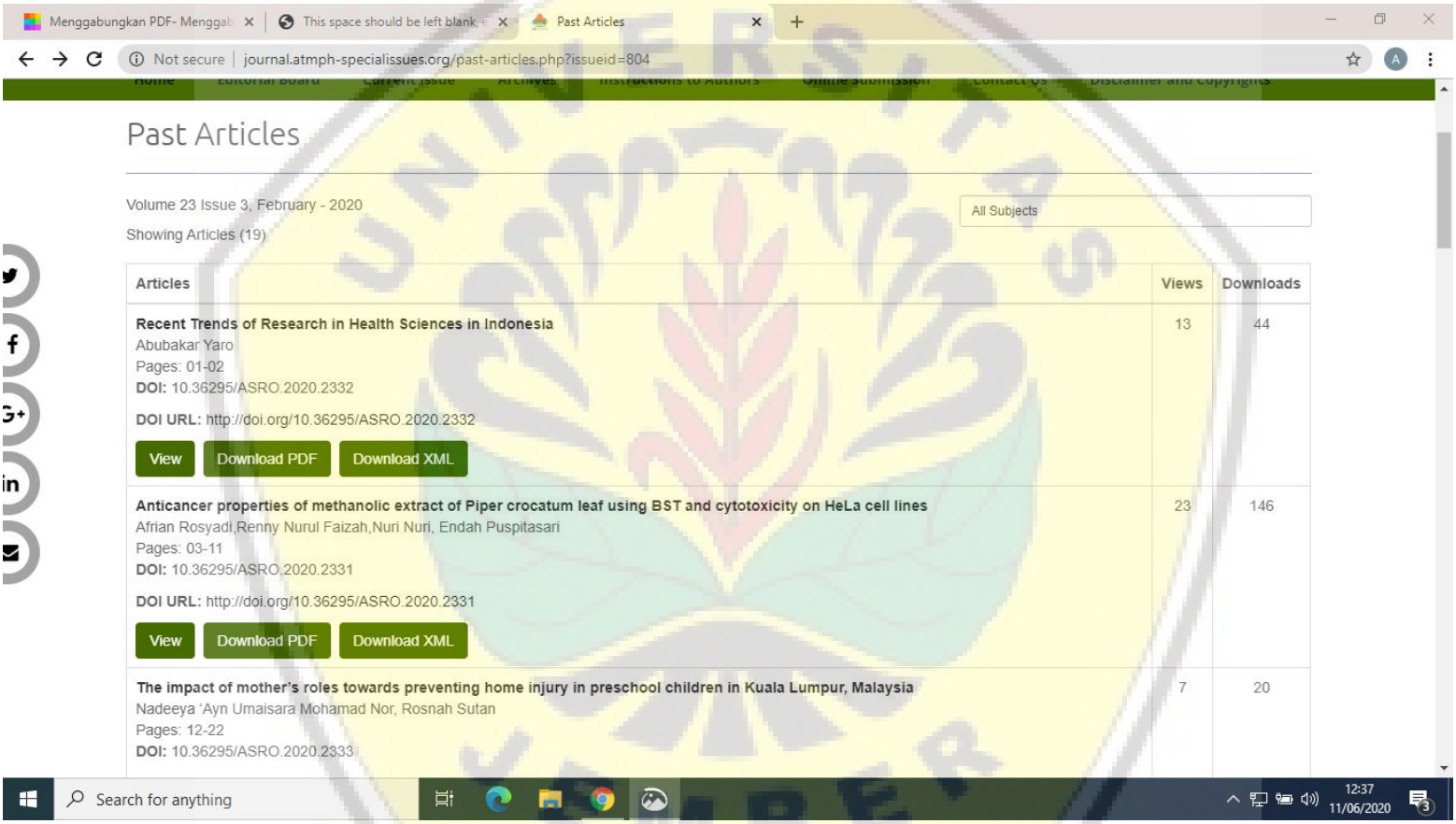

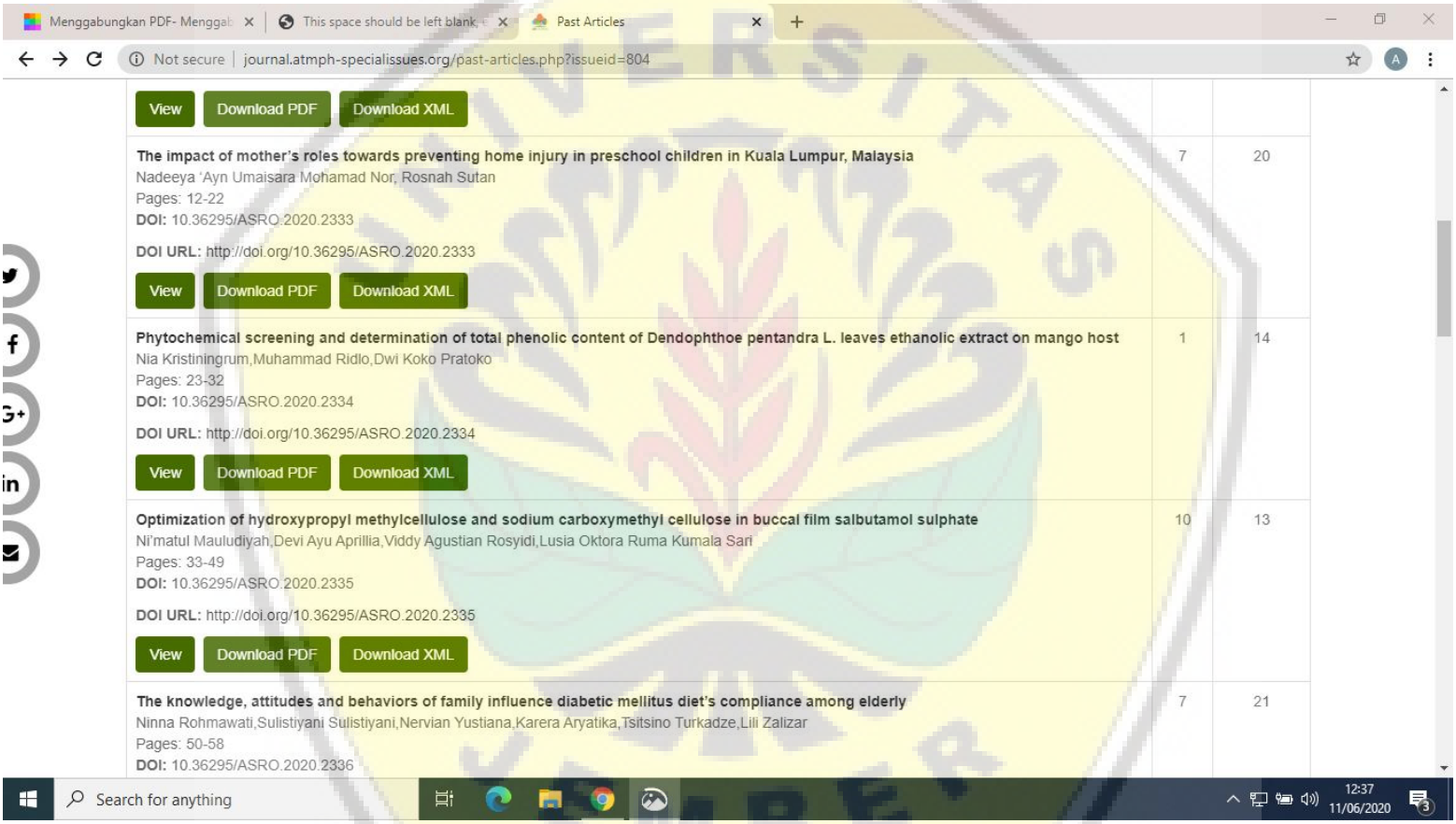

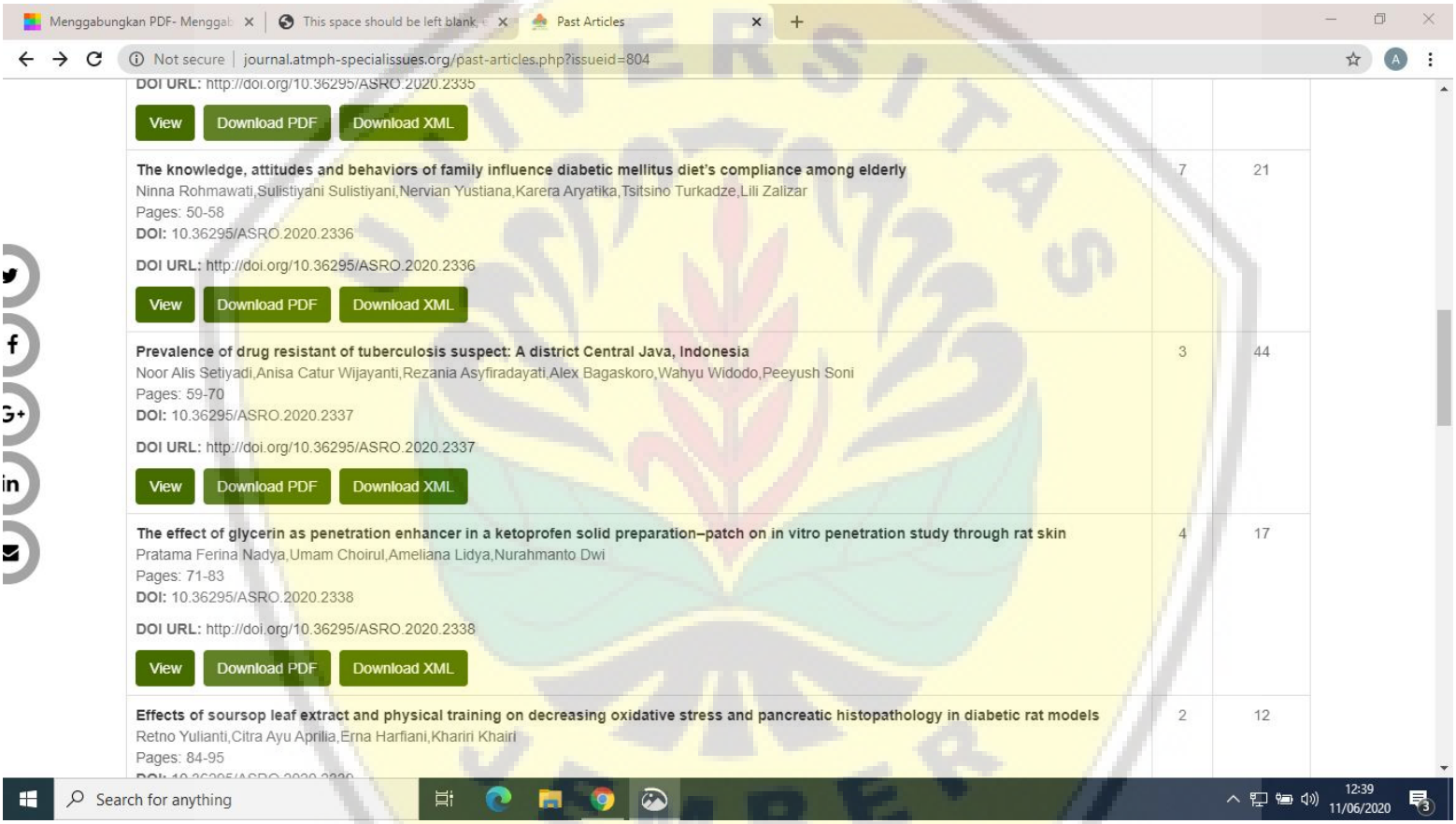

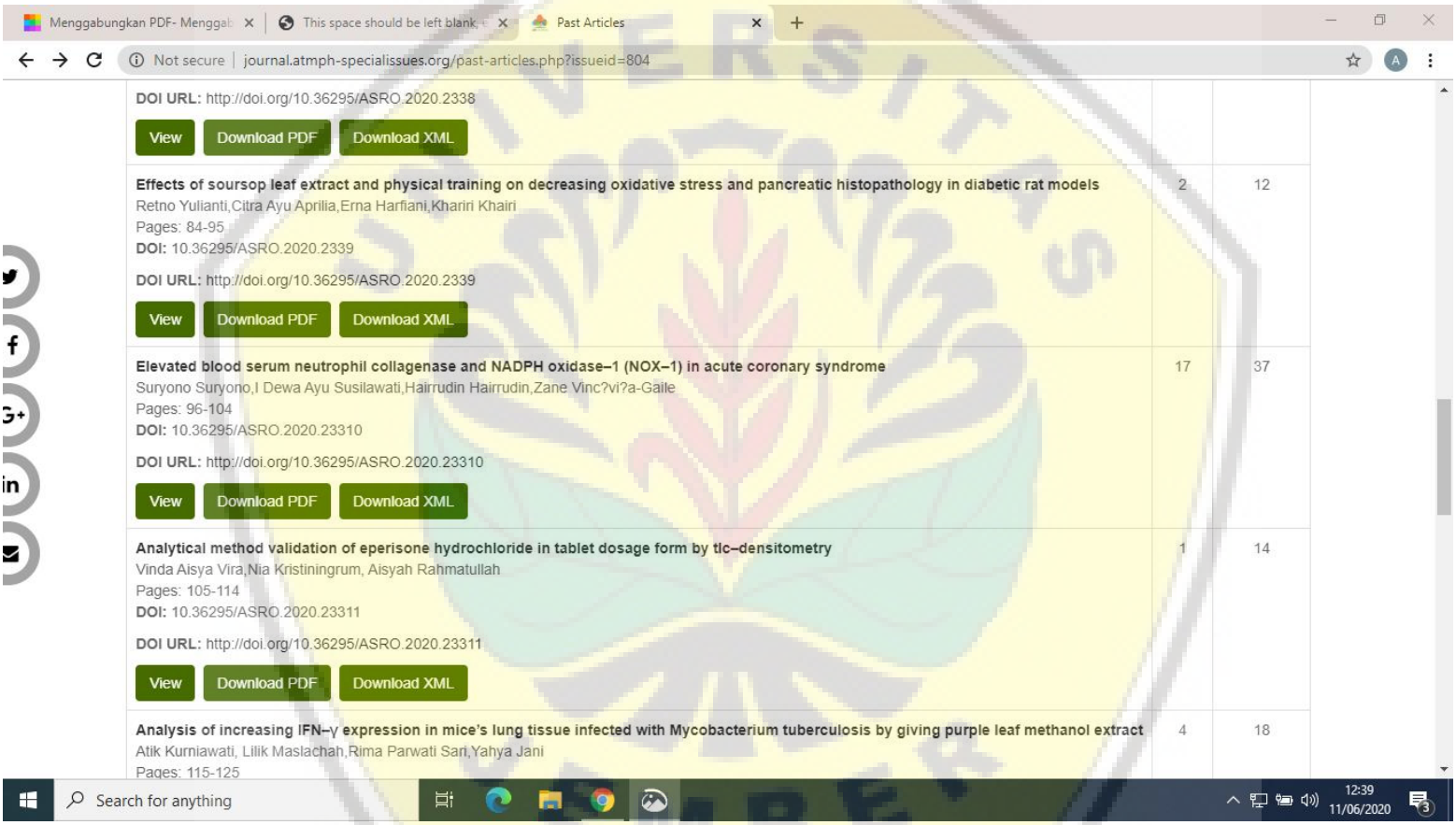

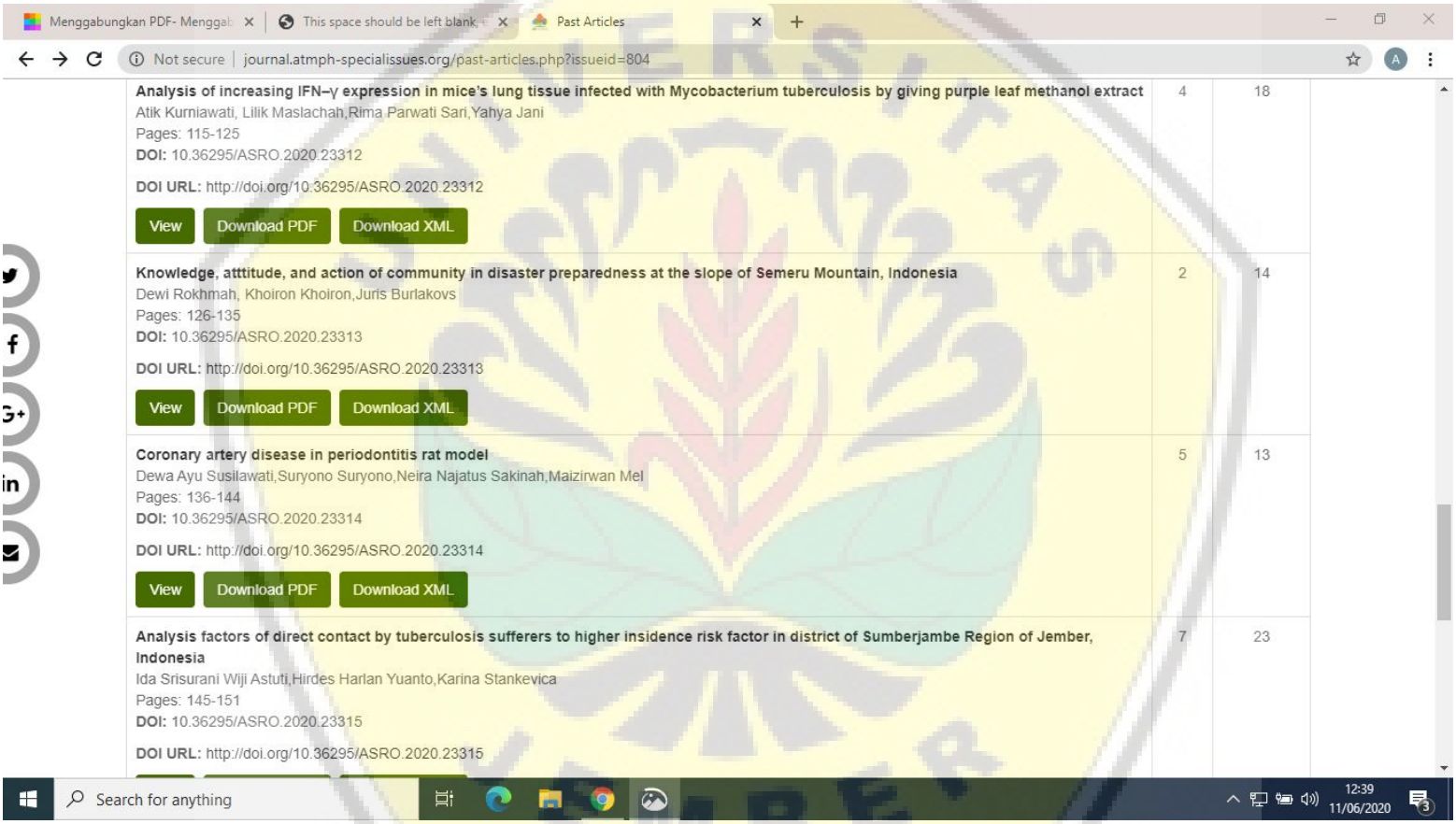

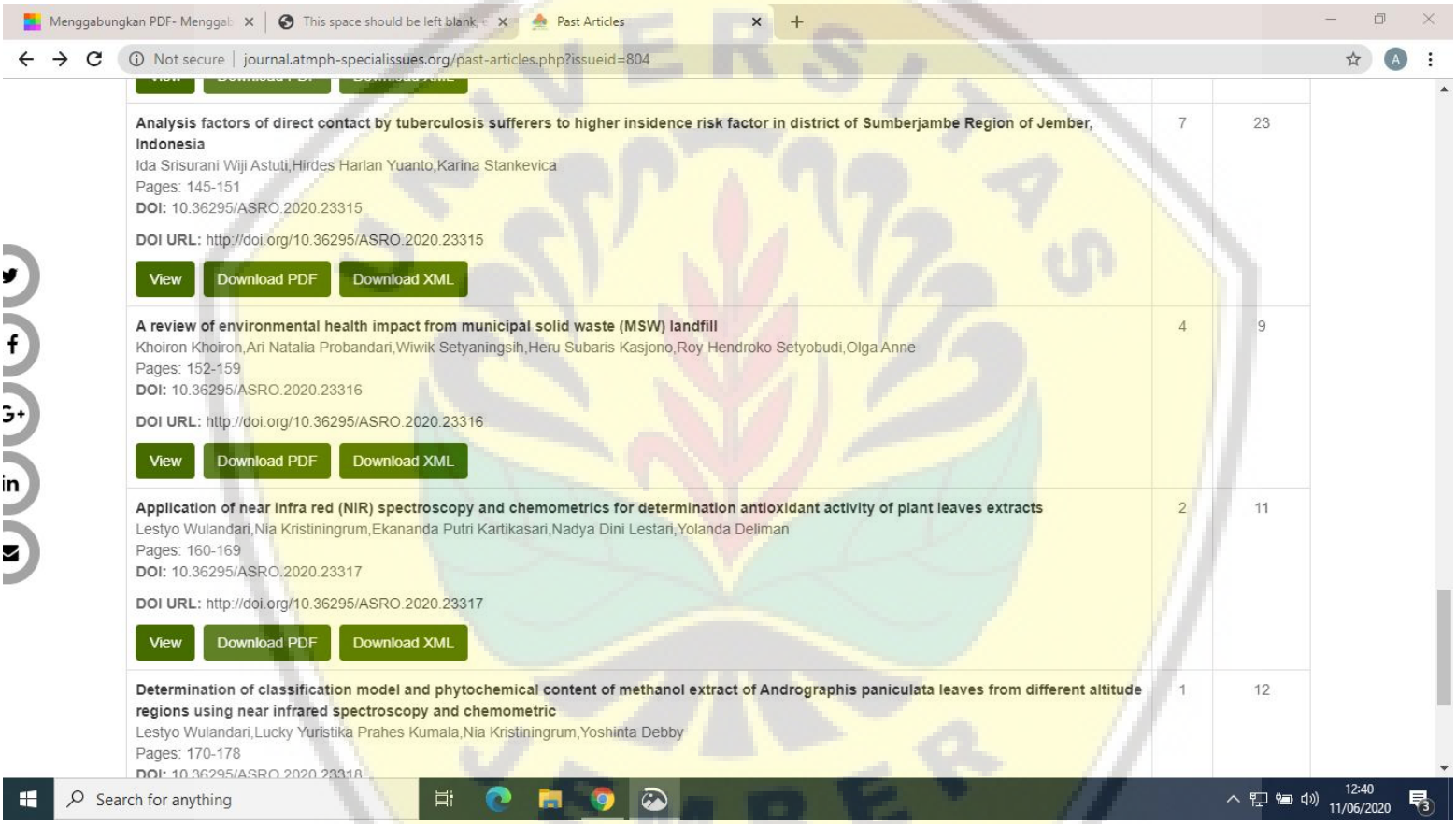

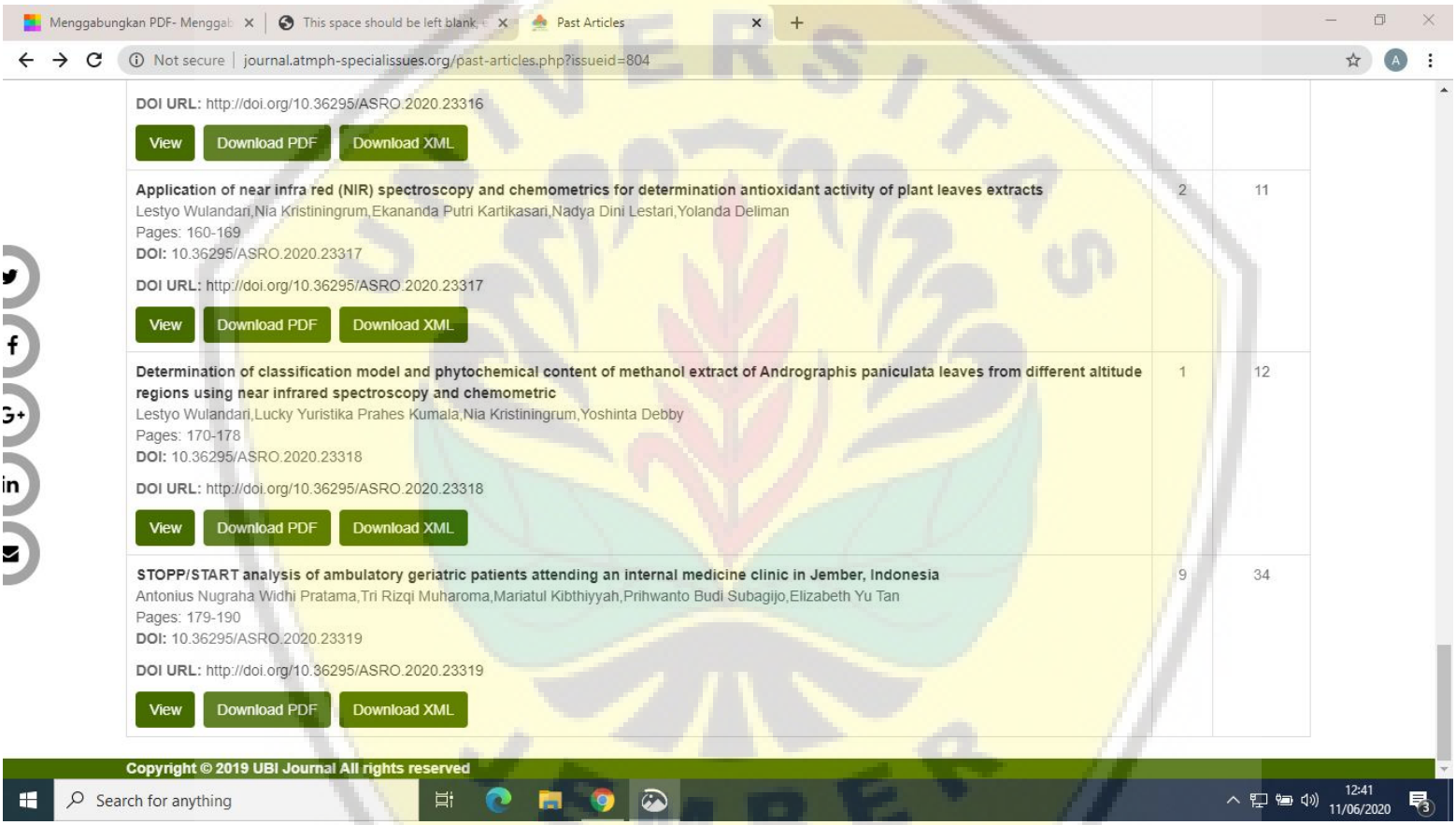

Analysis of increasing IFN–γ expression in mice's lung tissue infected with *Mycobacterium tuberculosis* by giving purple leaf methanol extract

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Abstract

Context: Tuberculosis is an infectious disease that highly depends on the immune response. Purple leaves (*Graptophyllum pictum* (L) Griff) has an immune–modulatory activity. **Aims**: The purpose of this study was to analyze the effect of purple leaf methanol extract (EMDU) on the expression of IFN–γ in mice lung tissue infected with *Mycobacterium tuberculosis*. **Materials and Methods**: *M. tuberculosis* was infected in mice. The EMDU was given with dose (1.703, 3.406, 6.812) mg kg^{-1} BW^{-1} for 14 d after infection. The expression of IFN–γ protein (expression obtained from mice's lung tissue) was examined using immunohistochemical examination using IFN–γ anti monoclonal antibodies. Calculations performed on immunoreactive cells showed positive expression and reddish–brown appearance on the cytoplasm. Calculated as many as ten fields of view using a light microscope at 400 times magnification, then the mean value is taken. The mean value of the number of immunoreactive cells is included as data. Data analysis by one way ANOVA and Duncan test. **Results**: The treatment group showed that IFN–γ expression in mice tuberculosis was significantly increased ($p < 0.05$) of EMDU. **Conclusions**: Administration of EMDU increase of IFN–γ expression in mice tuberculosis.

Keywords: *Graptophyllum pictum* (L) Griff, immune, tuberculosis.

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Introduction

Mycobacterium tuberculosis (Zopf, 1883) is an infectious disease that highly depends on the immune response. The severity of tuberculosis is mainly influenced by the host's immune response. Various theories suggest that innate immunity is the leading immune response, especially the most potential macrophages against *M. tuberculosis*[1]. Macrophages as professional phagocytes cells with the main function of destroying immunogens and as Antigen Presenting Cells (APC) recognize microbes through several receptors that are associated with their function to stimulate cell migration to the site of infection and stimulate the production of microbial substances. The accuracy of the recognition of these pathogens is influenced by receptors that affect the pathogen attachment in APC. It is suspected that one of the receptors that activate macrophages to stimulate innate immunity is Toll–Like Receptor (TLR) which will affect the Nuclear Factor Kappa Betha (NFkB) transcription factor, then stimulate phagocytic activity and cytokine production[2]. In tuberculosis, TLR–2 suppression is suspected to occur which results in a decrease in the function of phagocytosis and affects pro–inflammatory cytokines TNF–α and IFN–γ and also anti–inflammatory cytokines TGF–β1. Changes in cytokine levels affect the level of lung tissue damage[3].

According to WHO report, there are 22 countries that have a high prevalence of tuberculosis patients. Most sufferers were in Asia (55 %), Africa (30 %), Middle East (7 %), Europe (4 %), and America (3 %). So almost the whole world is not free from tuberculosis. The incidence of tuberculosis in Indonesia ranks number five in the world after India, China, South Africa, and Nigeria. The high incidence of tuberculosis shows that there is still a lack of success in handling tuberculosis. Efforts to control TB in terms of prevention, discovery of new cases and management of TB therapy are very necessary, The treatment efforts that have been carried out so far include the provision of Anti Tuberculosis drugs (OAT). Treatment with OAT, using more than one type of drug or combination of several drugs, and long–term use (at least 6 mo) makes treatment uneffective. The weakness is that the patient becomes disobedient, lazy or forget, and does not want to continue treatment. As a result, tuberculosis does not heal, the emergence of resistant *M. tuberculosis* becomes a dangerous source of transmission[4].

The problem of tuberculosis treatment is very complex, in addition to its long–time, the combination of anti–tuberculosis (OAT) drugs can also cause resistance. Some researchers use immunomodulators as additional therapy. In general, immunomodulators only affect one aspect of a complex immune response so that they are unable to generate various aspects of the immune response to eliminate bacteria[5]. Besides, immunomodulators are expensive and have side effects such as fatigue, flu–like syndrome, decreased appetite, loss of fertility, bone marrow suppression, depression to suicide, autoimmune and thrombocytopenia. For this reason, the use of cytokines as immunomodulators for the prevention of tuberculosis has not yet been implemented[6]. This fact prompted researchers to find immunomodulatory materials suitable for tuberculosis infection.

The purple leaf is one of the traditional Indonesian medicinal plants. Purple leaves (*Graptophyllum pictum* (L) Griff) are included in the list of 66 biopharmaceutical plant commodities which are stipulated through a Decree of the Minister of Agriculture Number: 511/Kpts/PD.310/9/2006. Indonesian people use this plant to treat swelling, burn, hemorrhoids and to launch menstruation[7]. Several studies have been conducted mentioning that the ethanol extract of purple leaves has anti–bacterial antimycobacterial activity against *M. tuberculosis* H37Rv in vitro[8], an anti–inflammatory effect[9], immune–modulatory on the function of phagocytosis and the formation of immunoglobulin M and TNF–α in mice[10]. The safety of purple leaves has been proven by several researchers through acute toxicity tests and subchronic toxicity tests. Purple leaf ethanol extract has a low acute toxicity value in mice given orally but should be investigated further for longer use[11]. Through a subchronic toxicity test for 3 mo, the administration of purple leaf ethanol extract in mice was declared safe and able to improve the survival of mice[12].

Based on scientific report data about the ability of purple leaves to fitopharmaca activity and see its long use in the community without causing side effects, the researchers want to analysis the effect of EMDU on the expression of IFN–γ in mice lung tissue infected with *M. tuberculosis*. The use of EMDU as an immunomodulatory material is expected to improve the function of phagocytosis of macrophages infected with *M. tuberculosis* so that the immune response can function again as a defense system to eliminate the bacteria.

Materials and Methods

This study is an experimental study in *Mus muculus (*Linnaeus, 1758) *Swiss Webster Strain* type Balb/c to compare between groups infected with *M. tuberculosis* with mice infected with *M. tuberculosis* that were given EMDU and groups of mice without treatment (normal). A total of 24 mices were used as experimental animals for pulmonary tuberculosis[13],[14]. All procedures for the treatment of the preparation of infected experimental animals in these mice have Research Ethics Feasibility with certificate No. 202.KE by Animal Care and Use Committee on Veterinary Medicine Universitas Airlangga Surabaya Indonesia. Before being infected, mice were anesthetized with ketamine intra–muscularly[14], Furthermore, mice are kept and given food and drink for 7 wk. The calculation of doses based on the dose of purple leaf immunomodulators is 0.2 mL infusion of 10 % po^{-1} day^{-1}[10]. Dose two of EMDU (3.406) mg kg^{-1} BW^{-1}, Dose three of EMDU (6.812) mg kg^{-1} BW^{-1}[10]. On the 29th d, mice in the treatment group were sacrificed for their lung tissue. The right lung tissue was taken aseptically, then was put into a 10 % formalin buffer for histopathological examination. The expression of TLR–2 protein (expression obtained from mice's lung tissue) was examined using immunohistochemical examination using anti–TLR–2 monoclonal antibodies. Calculations performed on immunoreactive cells showed positive expression and reddish–brown appearance on the cytoplasm. Calculated as many as ten fields of view using a light microscope at 400 times magnification, then the mean value is taken. The mean value of the number of immunoreactive cells is included as data[15],[16],[17]. The data statistical analysis using Statistical Package for the Social Sciences (SPSS) Statistics. Kolmogorov–Smirnov Normality test to find out the data is normally distributed ($p > 0.05$), followed by the Kruskal Wallis test and

Mann–Whitney u test. The Levene test is conducted to determine the homogeneity of the data. If the data variance is homogeneous ($p > 0.05$), it is followed by the ANOVA test. The analysis of the Duncan test which is to detect significant differences in each group of samples.

Result

The results of examination of immunohistochemical preparations showed that there was an increase in IFN–γ expression in treated lung tissue (K1, P1, P2 and P3). The mean IFN–γ expression in groups K0, K1, P1, P2 and P3 is seen in Table 1. The normality test with Kolmogorov–Smirnov for IFN–γ expression in all groups showed that the data were normally distributed ($p > 0.05$). Homogeneity test with Levene's test shows IFN–γ expression between groups has homogeneous variance ($p > 0.05$). The results of the F test in ANOVA between dose groups (1.703, 3.406, 6.812) mg kg^{-1} BW^{-1} showed significant differences ($p = 0.000$). This means increasing IFN–γ expression due to EMDU administration.

Table 1 Mean, standard differentiate and anova IFN–γ expression on mice's lung tissue

Groups	Mean (%)	D	Anova
Control (–)/(K0)	2.25[a]	1.59	F = 47.451
Control (+)/(K1)	6.42[a,b]	0.71	p = 0.000
P1 / (*M. tuberculosis*+D1)	9.08[b]	1.69	
P2 / (*M. tuberculosis*+D2)	12.00[c]	3.28	
P3 / (*M. tuberculosis*+D3)	12.50[c]	2.27	

**)The differential superscript on the same coloumn shown the real differentiation ($p < 0.05$)*

Note : K0 (without *Mycobacterium tuberculosis* + CMC); K1 (*Mycobacterium tuberculosis* + CMC); P1 (*Mycobacterium tuberculosis* + EMDU 1.703 mg kg^{-1} BW^{-1}); P2 (*Mycobacterium tuberculosis* + EMDU 3.406 mg kg^{-1} BW^{-1}); P3 (*Mycobacterium tuberculosis* + 6.812 mg kg^{-1} BW^{-1}).

To determine differences in IFN–γ expression between groups with doses (interaction treatment with doses) Duncan test was performed. The Duncan test results between groups showed that ($p < 0.05$) means that there were significant differences. Based on the description above, it means that the infection of *M. tuberculosis* causes an increase in IFN–γ expression, besides that EMDU administration also increases IFN–γ expression. Increased IFN–γ expression increased with increasing EMDU dose but increased IFN–γ expression was significant (significant) between K1 group (*M. tuberculosis* only) and P2 (EMDU dose 3.406 mg kg^{-1} BW^{-1}), K1 (*M. tuberculosis* only) with P3 (EMDU dose 6.812 mg kg^{-1} BW^{-1}), group P1 (EMDU dose 1.703 mg kg^{-1} BW^{-1}) with P2 (EMDU dose 3.406 mg kg^{-1} BW^{-1}) and P1 (EMDU dose 1.703 mg kg^{-1} BW^{-1}) with P3 (EMDU dose 6.812 mg kg^{-1} BW^{-1}).

Discussion

The results of the identification of the content of EMDU obtained in this study by thin layer chromatography showed positive results on terpenoids, flavonoids, alkaloids, anthraquinones and polyphenols. These results are in accordance with several studies that have identified compounds contained in purple leaves. The results can identify the presence of flavonoids, flavonols, flavononones and aurons from methanol extract of purple leaves n–butanol fraction. The results of the study have obtained a chromatogram profile from the hydrolysis of ethanol extract of purple leaves, where the peaks were similar to the standards of the flavonoids of Kaempherol and Myricetin types[10]. Although the results of research on purple leaves have been able to identify the active compounds contained in it, but in this study selected preparations in the form of extracts. Based on the research of Jiangseubchatveera, N., et al.[18], it was reported that administration of preparations in the form of extracts of EMDU to artificial anorectal ulcers in *Rattus norvegicus* rats showed that there was a decrease in ulcer lumps with the highest healing rate of 61.73 % compared to the administration of preparations in the form of juice or infusion. This illustrates that preparations in the form of extracts have much better than single compound such as terpenoids, flavonoids, alkaloids, anthraquinones and polyphenols caused extract have multi synergistic effect of the EMDU[18],[19].

In this study, the method of infection with *M. tuberculosis* in experimental animals was selected intra–tracheal. The safest way for intratracheal infection, both for researchers and for the preparation environment in the form of extracts has a multi–factor influence and the synergistic effects of various compounds contained in a surrounding plant, because bacteria enter the trachea directly. The advantages of intra–tracheal infections are that bacteria do not enter through the upper respiratory tract such as the pharynx or larynx. This will allow choking, because the presence of bacteria on the nasal mucosa is a foreign object. As a result of these reflexes, only a portion of the bacteria that enter the lung tissue and some are wasted out which can endanger the researcher and the surrounding environment. In addition, this method has proven to be capable and effective in causing infection and histopathological abnormalities in mice's lung tissue and can be used to assess the growth of CFU mL^{-1} colonies of *M. tuberculosis*[15],[20],[21],[22].

The experimental animals used in this study were Balb/c mice, because tuberculosis infection in mice produced a fast and specific immune response[22] which correlated with the immune response in humans[23]. In addition, it has been proven that mice have a description of the pathogenesis of human–like tuberculosis as well as histopathological features that occur due to tuberculosis infection which represent infections in humans[24]. Besides that mice have similarities on the basis of physiology, anatomy and immune response with humans[23]. In this study, lung tissue was taken for examination of IFN–γ expression in the right lobe, because of its larger size consisting of three waves of the left lobe consisting of two waves.

Increased IFN–γ expression in mice's lung tissue, occurred in groups of mice infected with *M. tuberculosis* (K1) and mice group infected with *M. tuberculosis* and

given Purple leaf ethanol extract (P1, P2 and P3). Increased expression of IFN–γ due to mice experiencing tuberculosis infection. This is in accordance with the results of another research, that cytokine profiles in mice infected with *M. tuberculosis* cause increased IFN–γ levels[24].

Increased IFN–γ expression in mice infected with *M. tuberculosis* was due to the presence of ligands from *M. tuberculosis* namely Lipoarabinomannan (LAM). LAM binds to receptors on the alveolar surface of macrophages, especially Toll Like Receptor–2 (TLR–2). TLR–2 binds to the Cluster of Differentiation–14 (CD–14) to recognize LAM. Bonding or recognizing of TLR–2 with components from LAM results in the formation of signals to activate immune cells or produce cytokines. Activation via TLR–2 results in the recruitment of the MyD88 cytoplasmic adapter. Furthermore, MyD88 interacts with IRAK 1 (IL 1–receptor associated kinases–1) and continues its signal to TRAF 6. TRAF 6 (TNF receptor assosiated factor 6) induces protein kinase complexes, resulting in IKK phosphorylation which activates the NF–kB transcription factor. IKKs that phosphorylate IkB cause the release of NF–kB to enter the nucleus and become gene transcription activators to secrete cytokines via the proinflammatory pathway. Increased expression of NF–kB causes an increase in the expression of pro–inflammatory cytokines, one of which is IFN–γ[25],[26],[27].

In addition, an increase in IFN–γ expression also occurs due to the administration of EMDU. This is presumably due to the effects of flavonoids from EMDU. The types of flavonoids that have been detected from purple leaves are flavonol quercetin, kaempherol, and myrecetin[10]. It is known that kaempherol is a compound that is capable of binding to estrogen receptors (ER)[28]. Macrophages have estrogen receptors[29], so that by giving EMDU, it is thought that a bond between kaempherol and estrogen receptors will occur on the surface of macrophage cells. The bond is expected to lead to intra–cellular signal delivery. The signal starts with increasing IkB activity bound to NF–kB. This results in degradation, increased IkB phosphorylation, and NF–kB translocation into the nucleus also increases. NF–kB will influence Naive T cells to increase the production of IFN–γ proinflammatory cytokines[29],[30],[31]. It is assumed that this mechanism can increase IFN–γ. In accordance with the results of a study from Leyva–López, N., et al.[32] that quercetin had an immunomodulating effect on T cells by increasing IFN–γ expression and decreasing the expression of cytokine IL–4 in Pheripheral Blood Mononuclear Cells (PBMC)[32]. An increase in quercetin concentration will be followed by an increase in IFN–γ expression. This is in accordance with the results of this study where the higher the EMDU dose is followed by an increase in IFN–γ expression, which increases IFN–γ expression because EMDU is linear.

IFN–γ is a cytokine that is important for defense against mycobacterial infections. IFN–γ release assay (IGRA) has been approved as a helping tool in diagnosing *M. Tuberculosis* infection. Individuals with low IFN–level levels show susceptibility to *M. tuberculosis* infection because IFN–γ is the main mediator for activating

macrophages against intracellular pathogens. Lack of IFN–γ will fail to maintain protective immunity against *M. tuberculosis*[33].

Conclusion

It was concluded that the increase in IFN–γ expression in this study was a result of the administration of EMDU containing various active compounds such as terpenoids, flavonoids, alkaloids, anthraquinones, and polyphenols, where preparations in the form of extracts had a multi–factor effect and synergistic effect from various compounds contained in these plants.

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