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IN VITRO ANTI-INFLAMMATORY ACTIVITIES OF FISSISPALLIN B ISOLATED FROM *FISSISTIGMA PALLENS*

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Summary

In vitro* Anti-Inflammatory Activities of Fissispallin B Isolated from *Fissistigma pallens

Inflammation refers to the process from the body against stimulation of infections, injuries, toxins, etc. Acute inflammation could become chronic with a category of other serious symptoms and diseases. Being a rich source of potential bioactivities, plants and their chemical components present a promising reservoir for developing new, safer anti-inflammatory drugs. *Fissistigma pallens* (Finet & Gagnep.) Merr. (Annonaceae), growing in the Northern regions of Vietnam has been used in traditional medicine to treat several diseases. In this study, the anti-inflammatory activity of fissispallin B (**FPB**) isolated from *F. pallens* was evaluated. Its nitric oxide (NO), tumor necrosis factor (TNFα), and interleukin 6 (IL-6) inhibitory activity and IL-10 stimulatory activity using LPS-induced RAW264.7 macrophage inflammatory model. The compound illustrated its promising NO inhibitory effects with IC_{50} values of 2.24 ± 0.17 μM. Besides, fissispallin B significantly expressed the inhibiting capacity on pro-inflammatory cytokines (IL-6, TNF-α) while it had not much effect on stimulation of anti-inflammatory IL-10 cytokine. Treatment of the fissispallin B at 2.5 μM and 5 μM in RAW 264.7 cells remarkably decreased NF-kB protein expression to 36.1% and 51.2% compared to that of the control, respectively ($P<0.05$) by using Western-blot analysis. On the other hand, the high percentage of cell viability indicated this compound had a weak cytotoxic effect in this experiment. In conclusion, fissispallin B showed potential *in vitro* anti-inflammatory bioactivity.

Keywords: *Fissistigma pallens, RAW264.7, Anti-inflammation, Nitric oxide, Fissispallin B (FPB).*

1. Introduction

Inflammation, which is composed of temporary (acute) or permanent (chronic) state, is a mechanism that protects the body from infections or injury [1]. Chronic inflammation is reported to contribute to many diseases such as heart attack, diabetes, etc. [2]. During the inflammatory response, tumor necrosis factor-α (TNF-α) induced vasodilation recruits more immune cells, such as monocytes and neutrophils, to the inflammation site [3]. The overproduction of TNF-α could result in disseminated intravascular coagulation, which leads to the failure of organs such as the liver, kidneys, heart, and lungs [4]. Interleukin 6 (IL-6) enhanced macrophage development and helped them and other leukocytes infiltrate the inflammatory site [4]. TNF-α and IL-6 were pro-inflammatory cytokines that could cause diseases such as asthma, other auto-immune diseases, and even cancer with an excess amount. Hence, the inhibition of these cytokines could control the inflammation. The primary biological function of IL-10 is limited to inflammatory responses. Additionally, IL-10 prevented the infiltration of macrophages into the inflammation site. Thus, IL-10 was a robust controller of inflammation [5]. Moreover, and as reported, Nuclear factor-κB (NF-κB) represents a family of inducible transcription factors, which regulates a category of genes involved in regulation of the inflammatory responses [6].

Many patients with inflammation were advised to consume non-steroidal anti-inflammatory drugs (NSAID), but they still have several adverse effects such as gastrointestinal

bleeding, hypertension, etc. [7]. Therefore, a novel compound that could show fewer adverse effects and still be effective and low cost was needed. *Fissistigma pallens* (Finet & Gagnep.) Merr. belonging to the Fissistigma genus distributed in the Northern regions of Vietnam, and has been used in traditional medicine for arthritis, rheumatism, and asthma treatments. According to previous reports, some bioactive compounds extracted from this plant, such as terpenoid and flavonoid, demonstrated many pharmacological activities such as anticancer [8],[9]. There were six novel compounds named fissispallins A-F, and one known compound [fissipallin] demonstrated the cytotoxicity activity in three human cancer cell lines [colon cancer (HT-29), skin melanoma cancer (A-2058), lung cancer (A-549)]. In the past and present, there have not been reports about the anti-inflammatory activity of compounds extracted from *F. pallens*. Hence, this article presents the anti-inflammatory activity of fissispallin B (FPB) isolated from this traditional herb.

2. Materials and methods

2.1. Chemicals and cell lines

Fissispallin B (FPB), the 99% pure compound isolated from *F. pallens,* was provided by Prof. N. X. Nhiem, Institute of Marine Biochemistry (VAST). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), antibiotics, glutamine, HEPES (Gibco, Thermo Fisher Scientific), trypan blue solution (Gibco), trypsin (Gibco), Dimethyl sulfoxide (DMSO) (Invitrogen, Thermo Fisher Scientific), Lipopolysaccharides (LPS), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), and other regular chemical agents are from Sigma-Aldrich (St.Louis, MO, USA). The RAW 264.7 cell line is from the Laboratory of Bioassay, IBT (VAST). The mouse TNF-alpha, IL-6, IL-10 DuoSet ELISA kits were purchased from the R&D System (Minneapolis, MN, USA). The antibodies were from Abcam (UK).

2.2. Cell culture

The RAW264.7 cells at the 3rd passage were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% antibiotics, and 1% G-glutamine, 1% HEPES at 37°C in a humidified 5% CO_2 atmosphere. After 2 days, the removed old culture medium was detached with Trypsin 0.05% for 5 minutes. After that, the cells were added to the new DMEM. The cells were sub-cultured with a ratio of 1/5 to 1/3 of the total cell.

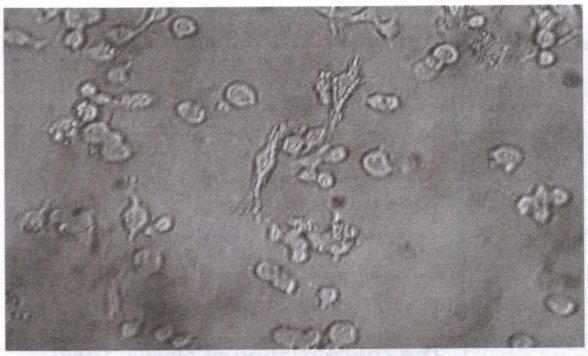


Fig. 3. RAW 264.7 cells at the day 3 of culture (Olympus microscope, 100X)

2.3. Cytotoxicity assay

The RAW 264.7 cells were seeded (1 x 10^4/well) in a 96-well plate and treated with 4 different concentrations of sample (10; 5; 2,5; and 1,25 µM), then incubated at 37°C, 5% CO_2 for 24 hours. Then, 20 µL of MTT (5 mg/ml) was added to each well, which was kept at 37°C for 4 hours. Afterward, the medium was removed, and the wells were rinsed with PBS 1X, followed by drying for about 2 hours and adding 200 µL of DMSO to dissolve formazan crystals. The absorbance was measured at 570 nm by the spectrometer [10]. The percentage of living cells was determined by the following formula:

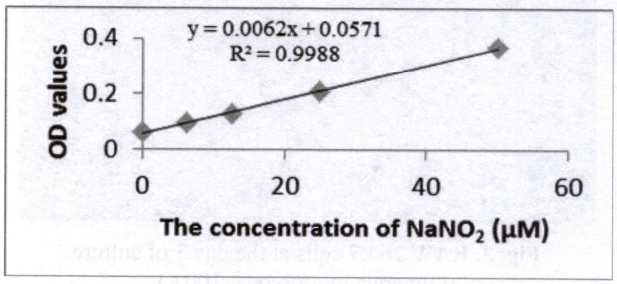
$$\%\ \text{Cell viability} = \frac{\text{OD(treated sample)}}{\text{OD(untreated sample)}} \times 100\%$$

2.4. Griess assay for nitric oxide production measurement

After being seeded and incubated, the culture medium of RAW 264.7 cells was replaced with a serum-free medium, and the cell was continuously incubated at 37°C and 5% CO_2 for 3h. The cell was then treated with different sample concentrations in 2h, followed by LPS stimulation (1 µg/mL) in 24h. There were untreated cells as the negative control. Dexamethasone was served as the positive control. After 24 hours of LPS incubation, 100 µL of supernatant was replaced into a new 96-well plate and reacted with 100 µL of Griess Reagent, including the equal volume of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride in water. The reaction occurred in 10 minutes at room temperature (RT), and a microplate reader measured the optical density of each well at 540 nm [11]. The NO_2^- concentration was calculated by using the standard curve of $NaNO_2$ and compared with negative controls according to the formula:

$$\% \text{ Inhibition} = 100\% - \frac{\% \text{ NO sample}}{\% \text{ NO negative control}} \times 100$$

The standard curve of $NaNO_2$ was as follows:



2.5. Determination of TNF-a, IL-6, and IL-10 production

RAW 264.7 cells were treated with FPB at 4 different concentrations with LPS stimulation (1 μg/mL), then incubated at 37°C, 5% CO_2, for 24 hours. The supernatants were collected and measured concentration of TNF-α, IL-6, and IL-10 following the procedure of the manufacturer of Mouse TNF-alpha, IL-6, IL-10 Duoset ELISA kits (R&D system, USA). The concentration of cytokines was calculated based on the standard curve for IL-6, IL-10, and TNF-α. The production of cytokines in the treated sample was compared with the untreated sample according to the formula:

$$\% \text{ Cytokine Production} = \frac{\text{Concentration of treated sample}}{\text{Concentration of untreated sample}} \times 100$$

2.6. Western blot analysis

RAW264.7 cells were treated with FPB at 2.5 μM and 5 μM concentrations for 48h and then rinsed in lysis buffer (RIPA). The supernatant was recovered by centrifuging the samples for 20 minutes at 12,000 rpm. Protein concentration was measured using the Bradford assay. For the Western blot, 30 μg of proteins from each sample was run on 12% SDS-PAGE gel, and the separated proteins were transferred onto the polyvinylidene fluoride (PVDF) membrane. The membrane was then blocked with 5% non-fat milk powder (w/v) at RT for 2 h, and the primary antibodies against NF-kB (1:1000) and GAPDH (1:200) were then incubated at 4°C overnight. After washing, the membrane was incubated at RT for 2 hours with the secondary antibodies (anti-mouse 1:10000). As an internal check for protein loading and transfer from the gel to the membranes, GAPDH was utilized. The Image J program was used to quantify the bands on the Western blot [12].

2.7. Statistical analysis

Each experiment in this study was performed in triplicate. The IC_{50} (inhibition concentration at 50%) values and the percentage of living cells with standard deviation (SD) were analysed using Microsoft Excel 2019, GraphPad Prism 8.0 software, and TableCurve 2Dv4 software.

3. Results

3.1. The impact of FPB on RAW264.7 cell growth and the ability of NO inhibition

Table 1 shows that the percentage of NO production was inhibited by FPB in the dose-dependent manner. FPB demonstrated strong inhibitory activity on NO synthesis with IC_{50} value at 2.24 ± 0.17 μM. The amount of NO inhibited by compound FPB at the concentration of 1.25 μM was relatively low compared to three other concentrations. Besides, the cytotoxicity assay by MTT was carried out simultaneously with the inhibition of NO production experiment to evaluate the cell viability. It could be seen that under the FPB treatment, the cell survival rate was around 85% to 100% at 4 concentrations. Therefore, those concentrations of FPB will be used in further experiments.

Table1. Effect of FPB on RAW264.7 cells and ability of NO inhibition

Conc. (μM)	FPB		Conc. (μM)	Dexamethasone	
	% NO inhibition	% Cell viability		% NO inhibition	% Cell viability
10	70.83 ± 2.55	85.63 ± 1.30	**100**	81.36 ± 2.31	95.18 ± 1.15
5	63.63 ± 4.24	89.34 ± 2.29	**20**	52.27 ± 0.94	98.56 ± 0.70
2.5	54.02 ± 2.55	95.71 ± 2.45	**4**	40.39 ± 1.86	99.62 ± 1.23
1.25	22.21 ± 2.09	101.22 ± 1.20	**0.8**	34.73 ± 1.59	103.33 ± 1.74
IC_{50}	**2.24 ± 0.17**		**IC_{50}**	**15.95 ± 1.05**	

3.2. The effect of FPB on the expression of TNF-α, IL-6, and IL-10

Further, the effect of FPB on the pro-inflammatory cytokine TNF-α and IL-6 production was assessed. The results show that, at the time of the study, at test concentrations of 10 μM and 5 μM, FPB effectively and significantly inhibited the production of TNF-α compared to the control LPS ($P<0.01$) (Table 2). FPB at concentrations of 1.25-2.5 μM did not show any inhibitory effect on TNF-α production.

Table 2. Expression of TNF-α by RAW264.7 cells under the influence of FPB

Sample	Concentration of TNF-alpha (ng/mL)			
	1.25 μM	2.5 μM	5.0 μM	10 μM
FPB	858.59 ± 20.57	845.23 ± 28.30	484.85** ± 10.28	441.42** ± 7.38
(+)LPS	878.98 ± 59.59			
(-)LPS	466.11 ± 21.35			

**P<0.05; **P<0.01 compared to LPS control*

In addition, table 3 showed that, for IL-6, under the treatment of FPB at concentrations of 10 μM and 5 μM, FPB effectively inhibited the production of IL-6 compared to the control LPS at a statistically significant (P<0.01). However, FPB at two lower concentrations did not show the inhibitory effects on the Il-6 production, similar to that on TNF-α level.

Table 3. Expression level of IL-6 by RAW 264.7 cells under the influence of FPB

Sample	Concentration of IL-6 (ng/mL)			
	1.25 μM	2.5 μM	5.0 μM	10 μM
FPB	151.24 ± 3.80	144.21 ± 5.22	139.48* ± 4.43	90.81** ± 4.15
(+)LPS	175.25 ± 11.25			
(-)LPS	57.96 ± 5,60			

**P<0.05; **P<0.01 compared to LPS control*

The result in table 4 showed that the expression of anti-inflammatory cytokine IL-10 treated with FPB at all concentrations was not significantly different in comparison with that of the LPS control (P>0.05).

Table 4. Expression level of IL-10 in RAW 264.7 cells under the influence of FPB

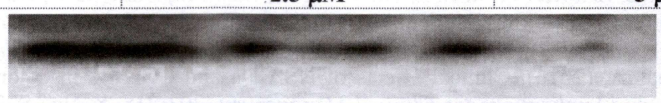
Sample	Concentration of Il-10 (ng/mL)			
	1.25 μM	2.5 μM	5.0 μM	10 μM
FPB	458.34 ± 50.04	480.65 ± 28.67	503.75 ± 19.53	514.59 ± 13.42
(+)LPS	496.95 ± 12.34			
(-)LPS	455.22 ± 13.19			

**P<0.05; **P<0.01 compared to LPS control*

3.3. FPB impacts the levels of NF-kB in RAW 264.7 cells.

Further, the effects of FPB at the two concentrations of 2.5 μM and 5 μM on marker NF-kB in RAW 264.7 cells were investigated (Table 5). As a result, the compound treatment significantly decreased NF-kB protein expression in treated RAW 264.7 cells at both 5 μM and 2.5 μM concentrations with a reduction of 51.2% and 36.1% compared to the control, respectively (P<0.01 and P<0.05).

Table 5. FPB at concentrations 2.5 μM and 5 μM impacts the levels of NF-kB in RAW 264.7 cells

	LPS	FPB 2.5 μM	FPB 5 μM
NF-kB			
GAPDH			
Expressed Level (%)	100	63.9*	48.8**

**P<0.05; **P<0.01 compared to LPS control.*

4. Discussion

Besides *F. pallens*, some other species of *Fissistigma*, such as *F. polyanthoides* and *F. acuminatissimum* Merr. also had attributed anti-inflammatory properties [13],[14]. The anti-inflammatory activities of different extracts of various plants belonging to the *Fissistigma* genus have been evaluated in plenty of previous reports. For example, Hu et al. (2007) reported 7-(3,4-Dihydroxyphenyl)-N-[(4-methoxyphenyl) ethyl] propenamide (Z23) (6.25; 25; 100 μM) had functioned in suppressing the production of nitric oxide, PGE2, cytokines (TNF-α and IL-6) from LPS-stimulated RAW264.7 macrophages [15],[16]. Additionally, ethanol crude extracts with $CHCl_3$ soluble part (1, 10, 25, 50 μg/mL) and alkaloids-2 (10 μM) from *F. oldhamii* (Hemsl.) Merr had shown a significant inhibitory effect on TNF-α and IL-6 induced by LPS-stimulated RAW264.7 cells [17]. More recently, the terpenoids 1-5 (IC_{50} 13.7 μM - 49.0 μM) from *F. polyanthoides* (A. DC.) Merr have reported the dose-dependent inhibitory impacts on NF-κB/AP-1 produced by LPS-stimulated THP1-Blue-CD14 cells [18]. Thereby, it could be seen that the *Fissistigma* genus in general, and *F. pallens* in particular with their crude extracts or isolated compounds, had the potential anti-inflammatory activity.

In this project, the anti-inflammatory activity of fissispallin B (FPB) isolated from *F. pallens* was evaluated on RAW 264.7 macrophage cells. Activation of macrophages by LPS could induce the inflammatory response, which releases pro-inflammatory cytokines including TNF-α and IL-6. Additionally, the expression of COX-2 and iNOS causes the production of various inflammatory mediators, including NO and PGE-2 [19]. The obtained results presented that FPB demonstrated strong inhibitory effects on NO production with the IC_{50} values 2.24 ± 0.17 μM. Further investigation revealed similar inhibitory effects on TNF-α and IL-6 production. The levels of those pro-inflammatory cytokines significantly decreased in the RAW 264.7 cells under 10 μM of FPB treatment. However, the compound did not show a remarkable effect on IL-10 stimulation. As a central factor of inflammation, NF-κB was also critically inhibited up to 51.2% reduction under 5 μM of FPB treatment.

According to the data, it could be seen that FPB had effective inhibitory activity on NO and pro-inflammatory cytokines. In contrast, the inducible activities of anti-inflammatory IL-10 and the toxicity on RAW 264.7 cells were insufficient.

5. Conclusion

In this report, FPB was evaluated for the *in vitro* anti-inflammatory activity on RAW264.7 macrophages. The results showed that FPB expressed vigorous activity in inhibiting nitric oxide with the IC_{50} values of 2.24 ± 0.17 μM. This compound also presented the significantly inhibitory activities of pro-inflammatory cytokines, including TNF-alpha and IL-6, while the toxic and promoting activity of this compound on anti-inflammatory cytokine IL-10 was weak. The compound also exhibited a strong capacity to reduce the expressed level of NF-κB, an important inflammatory mediated factor. In conclusion, *F. pallens* contained the potential bioactive compounds exerting anti-inflammatory activity.

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POSTPRANDIAL GLYCEMIC REGULATING EFFECT OF *ENSETE GLAUCUM* SEED EXTRACT

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Summary

Postprandial Glycemic Regulating Effect of *Ensete glaucum* Seed Extract

Recently, plant-based products have been given much attention as a cure for type 2 diabetes. Acknowledging the issue, the current study was aimed at evaluating the potential of *Ensete glaucum* seed extracts in postprandial glycemia management. *E. glaucum* seed extracts were examined in the *in vitro* α-amylase and α-glucosidase inhibitory assay to detect the potential extract. After that, an oral glucose tolerance test on mice was applied for the *in vivo* study to test the effect of the potential extract in postprandial glycemia management by measuring plasma glucose levels. The results illustrated that *E. glaucum* seed extracts had the ability to inhibit these enzymes, in which 96% ethanol extract by maceration with an IC_{50} value of 0.496 mg/mL in α-amylase inhibitory activity and 96% ethanol extract from hot extraction stood out from other extracts with an IC_{50} value of 1.511 µg/mL in α-glucosidase inhibitory activity. The hot-extracted 96% ethanol extract also had antioxidant activity *via* DPPH and ABTS inhibition with IC_{50} values of 10.98 µg/mL and 12.03 µg/mL, respectively. Seven-day administration of the hot-extracted 96% ethanol extract (50 mg/kg b. w., *p. o.*) significantly reduced the plasma glucose level in mouse oral glucose tolerance test. These preliminary findings suggested that *E. glaucum* seeds had potent anti-hyperglycemic activity, and contributed more in supporting diabetes regimes. Further studies need to be conducted to clarify the effect of *E. glaucum* seeds on diabetes.

Keywords: *Ensete glaucum seeds, Ethanol extract, α-Amylase, α-Glucosidase, Oral glucose tolerance test, Postprandial glycemia.*

1. Introduction

Type 2 diabetes, which is also previously known as adult-onset diabetes, non-insulin-dependent diabetes, accounts for roughly 90 to 95% of those living with diabetes. Half of people suffering from this chronic disease do not know their status [1]. In general, this chronic disease meditation is not needed for insulin treatment throughout a patient's lifetime; however, its consequences relate to the risk of developing macrovascular and microvascular complications [2]. In Vietnam, one in every 20 people is diagnosed with diabetes. By the end of 2015, the number of people with diabetes is up to 63 021 cases, about 5.6% of people 20 to 75 years [3]. In 2035, the incidence of diabetes and prediabetes