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Purification and Characterization of Fractions Containing Polysaccharides from *Talinum triangulare* and Their Immunomodulatory Effects

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Abstract: Previous studies identified that extracts of *Talinum triangulare* rich in flavonoids and phenolic acids showed antioxidative and immunomodulatory activities. In this study, the L9 orthogonal array was used to determine the optimal extraction conditions for water-extracted polysaccharides of *T. triangulare* (TTP) by hot reflux extraction and ultrasonic assisted extraction (UAE) methods. Results showed that while both extraction methods obtained a maximum polysaccharide yield of 3.1%, the optimal conditions for obtaining TTP was by UAE method. TTP was separated into large (LTTP) and small (STTP) molecular weights by dialysis. Since LTTP showed better effects than STTP in inducing macrophages to produce nitric oxide (NO) and indirectly inhibiting human cervical cancer HeLa cells, six different LTTP fractions were separated using anion-exchange chromatography. Contents of polysaccharides, triterpenoids, polyphenols, and proteins and molecular weights of major polysaccharide in each fraction were analyzed. The F1 fraction of LTTP, which showed the highest inducing ability of mouse RAW264.7 macrophages to secrete NO and tumor necrosis factor-α, showed the most significant indirect inhibitory effect of human colon cancer SW620 cells. These results suggest that LTTP, especially the F1 fraction, of *T. triangulare* may be used in health foods or Chinese medicine for its immunomodulatory potential.

Keywords: *Talinum triangulare*; Polysaccharides; RAW264.7 macrophages; immunomodulatory activity; infrared spectrum

Citation: Yeh, S.-H.; Hsu, W.-K.; Chang, Z.-Q.; Wang, S.-H.; Hsieh, C.-W.; Liou, G.-G.; Lee, H.-B.; Jiang, B.-H.; Tsou, H.-K.; Tsai, M.-S. Purification and Characterization of Fractions Containing Polysaccharides from *Talinum triangulare* and Their Immunomodulatory Effects. *Processes* **2021**, *9*, 709. https://doi.org/10.3390/pr9040709

Academic Editor: Bonglee Kim

Received: 15 March 2021
Accepted: 14 April 2021
Published: 16 April 2021

1. Introduction

Several herbal preparations are used to enhance the body's immune status. Many plant constituents, including saponins, glycosides, polysaccharides, alkaloids, flavonoids, sterols, and sterolins, have the ability to modulate the immune system [1]. Polysaccharides extracted from plants and fungi have recently drawn more attention from researchers and consumers due to their antitumor activities and relatively low toxicity [2,3]. Therefore,

the discovery and evaluation of polysaccharides with antitumor and immunostimulating properties has emerged as an important research field in chemistry and biology [4].

Talinum triangulare, known as "cariru" or "waterleaf", originates from tropical rainforest regions and is widely grown as a medicinal and food crop in western Africa, Asia, and South America, especially in Nigeria. Several articles have shown that *T. triangulare* can increase stamina and function as an immune-stimulant [5–8]. In Taiwan, *T. triangulare* is used as traditional medicine in the treatment and prevention of hepatic ailments and cancer. It has been found that *T. triangulare* polysaccharides (TTP) possess significant antioxidant [7–12], hepatoprotective [12], anti-hypercholesterolemic [13], anti-diabetic [14,15], and anti-tumor activities [7,8,16]. We previously investigated the antioxidant activities of various *T. triangulare* extracts, whereby the extracts exhibited significant antioxidant activities due to the presence of flavonoids and phenolic acids. All conditioned media obtained from human mononuclear cells cultured with various *T. triangulare* extracts showed significant inhibition (more than 40%) of U937 cell growth, indicating immunomodulatory potential [7].

In this study, we aimed to optimize the extraction process of water-soluble polysaccharides from *T. triangulare* (TTP) using the L_9 orthogonal array, in which two extraction methods with three main factors (extraction time, extraction temperature and ratio of raw material to water) were chosen as extraction parameters. In addition, TTP were purified using anion-exchange chromatography and their immunomodulatory and antitumor activities were evaluated *in vitro*.

2. Materials and Methods

2.1. Samples and Chemicals

The leaves of *T. triangulare* were collected from Wufeng (Taichung, Taiwan) as previous report [7], washed, freeze-dried, and then powdered. All other reagents and solvents used were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Double-distilled water was used throughout the experiments. The mouse macrophage cell line (RAW264.7, BCRC 60001), human cervical cancer cell line (HeLa, BCRC 60005), and human colon cancer cell line (SW620, BCRC 60343) were purchased from the Bioresources Collection and Research Center, Hsinchu, Taiwan. The media and culture conditions of these three cell lines were prepared according to manufactural instructions.

2.2. Extraction of Polysaccharides of T. triangulare (TTP) by the Heat Reflux Extraction (HRE) Method

The dried leaf powders were extracted under conditions using the L_9 orthogonal array. Two grams of dried leaf powders extracted with hot water were kept at 75 °C, 85 °C and 95 °C; the extraction times were 30, 60, 90, 120 and 150 min, and the ratio of material to water were 1/15, 1/20, 1/25, 1/30 and 1/35 g/mL by Rotavapor (R-3000, BUCHI Labortechnik AG, Flawil, Switzerland). After filtration, extracts were added with 4 volumes of 95% ethanol (1:4, v/v) and kept at 4 °C overnight. The TTP precipitates were obtained after centrifugation at 6000× g for 20 min and then lyophilized. The polysaccharide yield (%) was calculated using the following Equation (1):

$$\text{Polysaccharide yield (\%)} = \text{Polysaccharide weight (g)}/\text{Raw material weight (g)} \times 100 \quad (1)$$

2.3. Extraction of TTP by the Ultrasonic Wave-Assistant Extraction (UAE) Method

The TTP extraction parameters in the UAE method were similar to those of the HRE method. For this method, the TTP was extracted by ultrasonic cleaner (DC600H, DELTA, Hi Sun Instrument Co., Ltd. Taoyuan, Taiwan). The constant power of 600 watt (W) were set up for all extractions.

2.4. Cell Assays

2.4.1. TTP-Treated RAW264.7 Media and Nitric Oxide (NO) Assay

Ten milliliters of extracted TTP were added into 40 mL of 95% ethanol overnight at 4 °C. After centrifuging at 6000× *g* for 20 min to remove the supernatant, the pellets were dialyzed at room temperature by dialysis tubing with a molecular weight cut-off (MWCO) of 12–14 kDa (D9402). The polysaccharides within the dialysis tube (the large Mw polysaccharides of *T. triangulare*; LTTP) and polysaccharides outside the dialysis tube (the small Mw polysaccharides of *T. triangulare*; STTP) were concentrated and their glucose concentrations determined using the phenol-sulfuric acid method [17].

RAW264.7 macrophages (7.5×10^5 cells) were cultured in 6-well culture dishes, and equal doses of LTTP or STTP (100 μg/mL), and phosphate-buffered saline (PBS) or lipopolysaccharides (LPS; 1 μg/mL) were added into media and cultured for 24 and 48 h. Cell media were filtered to obtain various condition media (CMs). The nitrite contents in these CMs were measured by Griess reagent using $NaNO_2$ as a standard [18].

2.4.2. HeLa Cell Survival Rates Cultured with Various RAW264.7 CMs

HeLa cells (2×10^4 cells) were cultured in 12-well culture dishes. After 24 h, cell media was removed, 1 mL of CMs pre-treated with LTTP, STTP, PBS and LPS and 1 mL fresh medium were added, and then cells were cultured for 48 h. After the addition of 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL), cells were incubated for 4 h. The survival rate of cells was measured by an ELISA reader (LT-4500, Labtech International Ltd., Heathfield, UK) at a wavelength of 570 nm using survival of PBS-treated CM designed as 100%.

2.4.3. Cell Survival, Nitrite and Cytokine Productions in Different Fractions of LTTP-Treated RAW264.7 CMs

RAW264.7 macrophages (7.5×10^5 cells) were cultured in 6-well culture dishes, and the equivalent amount of PBS, LPS and different fractions of LTTP (100 μg/mL) were added and cultured for 24 h. The cell viabilities for different treatments were determined by MTT assay. After centrifugation, the supernatants of cultured media with different treatments were collected and filtered to obtain different condition media (CMs). The nitrite concentrations in different CMs were determined using the Griess reagent. The concentrations of tumor necrosis factor-alpha (TNF-α) in CMs were determined using a commercial enzyme-linked immunoassay (ELISA) kit (Bender MedSystems, Inc., Burlingame, CA, USA).

2.4.4. SW620 Cell Survivals Following Cultured with RAW264.7 Condition Media Pre-Treated with LTTP Fractions

SW620 cells (3×10^5 cells) were cultured in 12-well culture dishes. After 24 h, cell media was removed and 1 mL of CMs treated with different fractions of LTTP and 1 mL of fresh medium were added, and then cultured for 48 h. The survivals of SW620 cells were measured by MTT assay. The cell survival rates of different experimental groups were compared with the control group (PBS-treated CM), which was designated as 100%, using the following Equation (2):

$$\text{Cell viability (\%)} = (A_{570} \text{ of different fractions of LTTP-treated CM} / A_{570} \text{ of PBS-treated CM}) \times 100 \quad (2)$$

2.5. Isolation and Purification of LTTP by Anion-Exchange Chromatography

LTTPs were injected into the ion exchange chromatography column (DEAE Sepharose Fast Flow, DCL6B100, 38 × 400 mm) and then water and 0.1–1 M NaCl were used to elute fractions at room temperature, respectively. Every 10 mL of eluted fractions was collected in a separate tube. After measuring glucose and protein concentrations, different eluted fractions were pooled then concentrated. After dialysis using water for three times at 4 °C for 3 days, different fractions were stored at −20 °C until use. The glucose concentration in each fraction was measured using the phenol-sulfuric acid method and the amount of

protein was determined by the Bradford method using fetal bovine serum (BSA) as the standard [19].

2.6. Analysis of LTTPs

2.6.1. Chemical Composition

The glucose concentrations of LTTPs were determined by the phenol–sulfuric acid method, using glucose as the standard. Total polyphenols were determined by Folin-Ciocalteu's reagent, using gallic acid as the standard [20]. Total triterpenes were determined by the vanillin-glacial acetic acid method, using ursolic acid as the standard [21]. Total protein was determined by the Bradford method using BSA as the standard.

2.6.2. Determinations of Mw of Polysaccharides

The Mw of the polysaccharides was determined by high-performance gel-filtration chromatography (HPGFC), using a Jasco HPLC system with one PSS SUPREMA Ultrahigh analytical column (300 mm × 8 mm), a UV detector (Jasco UV-2075 Plus) at 280 nm, and an RI detector (Jasco RI-2031 Plus). The mobile phase used was a 0.2 mg/mL NaN_2 buffer and the flow rate was 0.8 mL/min at 30 °C. The samples were dissolved in a 0.1 M Na_2HPO_4 buffer. The sample volume (1.0 mg/mL) injected was 20 µL. The Mw was estimated by reference to a calibration curve made from a set of dextran standards of known Mw; 5, 12, 25, 50, 80, 150, 270, 410, 670 and 1400 kDa.

2.7. Infrared (IR) Spectroscopy Analysis

The IR spectrums of different fractions of LTTPs were determined using a Fourier-transform IR spectrophotometer (FTIR-8400S, Shimadzu, Japan) equipped with Shimadzu IR solution 1.30 software. The purified fraction was ground with KBr powder and then pressed into pellets for FTIR measurement in the frequency range of 400–4000 cm^{-1}.

2.8. Statistical Analyses

All experiments were performed at least in triplicate and the results were expressed as mean ± standard deviation (SD). Data obtained were analyzed using a one-way analysis of variance (ANOVA; SPSS, version 16.0), and Duncan's multiple range test. p values of less than 0.05 were considered to be statistically significant.

3. Results and Discussion

3.1. Optimal HRE Condition for TTP Extraction

Quantitative and qualitative studies of bioactive compounds from plant material mostly rely on selecting the proper extraction method because it plays a crucial role in outcomes [22]. The polysaccharide yields of TTP extracted with HRE under different conditions using L9 orthogonal design were shown in Table 1. The polysaccharide yields were increased as temperature increased and were directly proportional to extraction time. When the material to water ratio reached at least 1/25, the polysaccharide yield was the highest (3.01%). No significant difference in polysaccharide yields was observed for the material to water ratio 1/30. According to the R value (R = 0.900 > 0.433 > 0.133), temperature had the greatest influence on the polysaccharide yield of TTP. Therefore, the factors, in decreasing order of influence on polysaccharide yield, were: extraction temperature > extraction time > the ratio of material to water. The optimal extraction conditions of HRE were determined as 95 °C, 150 min, 1/25, and the highest extraction ratio of TTP was 3.01%. A previous study indicated that the yield of polysaccharides was 14.99% under optimal conditions for extracting soluble polysaccharides from T. triangulare using hot water extraction, which were as follows: extraction temperature of 100 °C, extraction time of 2 h, material to water ratio of 1/25 (g/mL), and two extraction cycles (repeated twice) [16]. Although the two extraction methods had different yields of polysaccharides, it is necessary to increase the extraction temperature and time in order to increase the yield of polysaccharides, which is a challenging and energy consuming.

Table 1. Yields of polysaccharides of *T. triangulare* extracted with HRE method under different conditions indicated using L9 orthogonal design.

Group	Temperature (°C)	Time (min)	Ratio of Material to Water (g/mL)	Yield (%) [a]
1	75	90	1/20	1.78 ± 0.06
2	75	120	1/25	2.03 ± 0.05
3	75	150	1/30	2.04 ± 0.06
4	85	90	1/25	1.82 ± 0.07
5	85	120	1/30	2.32 ± 0.08
6	85	150	1/20	2.73 ± 0.07
7	95	90	1/30	2.82 ± 0.09
8	95	120	1/20	2.73 ± 0.10
9	95	150	1/25	3.01 ± 0.12
K_I	5.79	6.40	7.20	
K_{II}	6.80	7.10	6.80	
K_{III}	8.50	7.70	7.10	
R	0.900	0.433	0.133	

[a] All values (n = 3 for all test groups) are expressed as mean ± SD of each group.

3.2. Optimal UAE Condition for TTP Extraction

UAE method has been employed to extract polysaccharides from different biological materials in the last few decades [23]. The yields of TTP extracted with UAE under different conditions using L9 orthogonal design were presented in Table 2. Essentially, a higher ultrasound frequency for a shorter time and lower ratio of material to water gave higher yields of TTP. We set up the power of 600 W for extraction. When the material to water ratio reached 1/20, the polysaccharide yield was the highest (3.14%). No significant difference in polysaccharide yield was observed for the material to water ratio 1/25. According to the R value (R = 0.633 > 0.533 > 0.133), temperature has the greatest influence on the extraction yield of TTP. The factors, in decreasing order of influence on polysaccharide yield, were: extraction temperature > ratio of material to water > extraction time. The optimal extraction conditions of UAE were determined as ultrasonic power of 600 W, 85 °C, 75 min, 1/20, and the highest extraction ratio of TTP was 3.14%.

Table 2. Yields of polysaccharides of *T. triangulare* extracted with UAE method under different conditions indicated using L9 orthogonal design.

Group	Temperature (°C)	Time (min)	Ratio of Material to Water (g/mL)	Yield (%) [a]
1	75	45	1/15	1.78 ± 0.03
2	75	60	1/20	2.27 ± 0.05
3	75	75	1/25	2.32 ± 0.06
4	85	60	1/15	2.41 ± 0.08
5	85	75	1/20	3.14 ± 0.09
6	85	45	1/25	2.78 ± 0.08
7	95	75	1/15	2.23 ± 0.11
8	95	45	1/20	2.64 ± 0.10
9	95	60	1/25	2.82 ± 0.11
K_I	6.43	6.41	7.25	
K_{II}	8.30	7.99	7.44	
K_{III}	7.53	7.87	7.87	
R	0.633	0.533	0.133	

[a] All values (n = 3 for all test groups) are expressed as mean ± SD of each group.

ANOVA analyses of the TTP yields indicated no significant difference between HRE and UAE methods. However, it should be noted that the extraction time of UAE was only half of HRE, and both of the extraction temperature and material to water ratio of UAE were lower than those of HRE. Huang et al. [24] reported that the optimal UAE conditions were obtained as follows: ultrasonic power 180 W, extraction temperature 80 °C, ultrasonic time

40 min, and material to water ratio 1/20 (g/mL). Under these conditions, the average yield of the polysaccharides was 20.01% with a relative standard deviation of 0.33%. Therefore, using UAE method to extract TTP is simple, fast and effective. In the following experiments, we used TTP obtained from our optimal extraction conditions by UAE method.

3.3. Cell Culture and Nitrite Productions of TTPs

The extracted TTP was precipitated by ethanol. After centrifugation, the pellets of TTP were dialyzed by dialysis tubing with a MWCO of 12–14 kDa. The polysaccharides within the dialysis tube (large TTP, LTTP) and polysaccharides outside the dialysis tube (small TTP, STTP) were obtained. The NO released by macrophages into culture media was measured as an indicator of immune-enhancing activity of alginates. Macrophage-derived NO can cause cytostasis or kill tumor cells in vitro [25]. As NO is an important cellular messenger for many physiological responses including inflammation, we were interested in whether treatments with 100 μg/mL LTTP or STTP induced NO productions in RAW264.7 murine macrophages. As shown in Figure 1A, NO (nitrite) concentrations in media were significantly increased in the 1 μg/mL LPS-treated group (43.5 ± 1.0 μM) compared to the PBS-treated control group (2.5 ± 0.1 μM). Nitrite concentrations in both the LTTP-treated (37.5 ± 1.0 μM) and STTP-treated (17.5 ± 0.5 μM) media were also significantly increased compared to the PBS-treated group. Furthermore, the LTTP-induced NO productions were significantly higher than that of the STTP-induced.

In this study, both the LTTP and STTP stimulated NO productions of RAW264.7 macrophages. We next tested whether both the LTTP- and STTP-treated macrophage CMs could inhibit human HeLa cell growth. The results, as shown in Figure 1B, demonstrated that CMs prepared by all of the LTTP, STTP and LPS pre-treatments significantly inhibited the growth of HeLa cells compared to the PBS-pretreated CM. The CMs cultured with 100 μg/mL LTTP and STTP for 48 h showed an inhibitions of HeLa cell growth of 93% and 87%, respectively. It can be concluded that TTPs are capable to stimulate RAW264.7 macrophages for secreting NO, and the TTP-treated CMs can significantly inhibit HeLa cell growth. Since the STTP and LTTP in the CMs were not washed out, the inhibitory effects of CMs for HeLa cell growth were in part by STTP or LTTP. From these results of NO productions and inhibitory effects of human HeLa cells, LTTP is more effective than STTP.

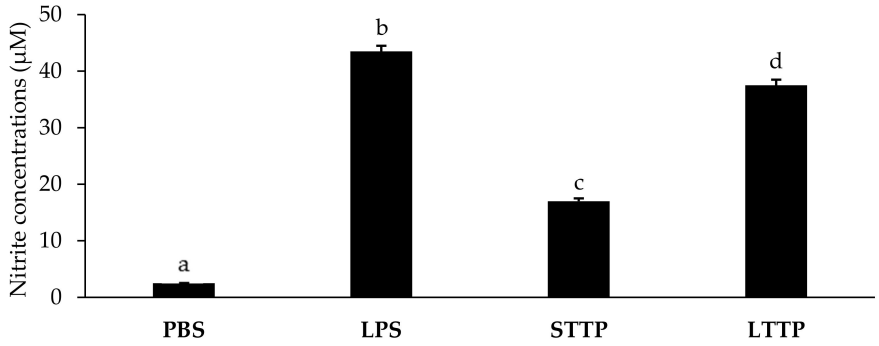
3.4. Separation and Fractionation of LTTP

To purify and determine the effective components of LTTP, we separated LTTP into different fractions using anion-exchange chromatography. LTTP was injected into a DEAE-Sepharose Fast Flow column and then eluted by water and 0.1–1M NaCl step gradients. The fractionation of LTTP resulted in six main fractions as defined, namely F1 to F6 (Figure 2). The amounts of polysaccharides (blue line) and proteins (red line) were mainly concentrated in F1, F2, F3, F4 and F6. Because of the low concentrations of both polysaccharides and proteins, F5 was excluded from further studies. Therefore, the components and immunomodulatory effects of F1–4, and F6 were analyzed.

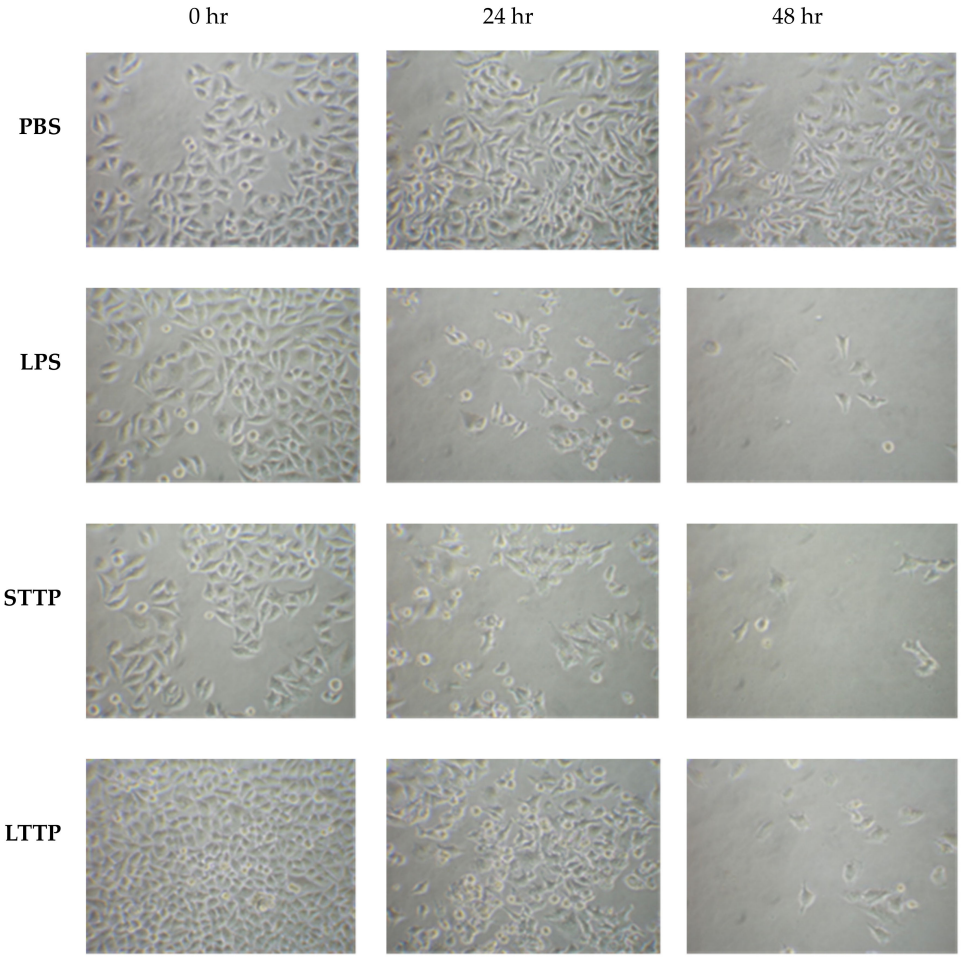
3.5. Chemical Components of Different Fractions of LTTP

Triterpenoids have been reported to possess hepatoprotective, anti-hypertensive, hypocholesterolemic and anti-histaminic effects, as well as anti-tumor and anti-angiogenic activities, and effects on platelet aggregation and complement inhibition. Polysaccharides, especially β-D-glucans, have been known to possess anti-tumor effects through immunomodulation and anti-angiogenesis. In addition, polysaccharides have a protective effect against free radicals and reduce cell damage caused by mutagens [26,27]. It is well known that polyphenol compounds belong to the bioactive components of plant products and have good health-promoting activities [28]. In this study, tubes of each of the F1 to F6 fractions were pooled, concentrated and then dialyzed by water. Table 3 showed the polysaccharide, triterpenoid, polyphenol and protein contents of the five different LTTP fractions excluding F5. Polysaccharide content, in decreasing order of amount, was F3

(25.00%) > F2 (21.52%) > F1 (12.67%) > F4 (5.77%) > F6 (0.67%). Triterpenoids content, in decreasing order of amount, was F3 (41.33%) > F4 (38.08%) > F6 (9.56%) > F1 (8.67%) > F2 (7.33%). Polyphenol content, in decreasing order of amount, was F4 (18.08%) > F1 (15.33%) > F3 (13.33%) > F6 (8.44%) > F2 (2.38%). The protein concentrations in the different LTTP fractions were very low accounting for 0.02–0.04% of all ingredients. Furthermore, it is worth noting that the unknown content, in decreasing order of amount, was F6 (81.29%) > F2 (68.76%) > F1 (63.31%) > F4 (38.05%) > F3 (20.30%).



(**A**)



(**B**)

Figure 1. Nitrite concentrations in mediums of RAW264.7 macrophages treated with phosphate-buffered saline (PBS), 1 μg/mL LPS (LPS), 100 μg/mL STTP (STTP), and 100 μg/mL LTTP (LTTP) for 24 h (**A**). Values (mean ± SD, n = 3 for all test groups) in each column not sharing the same alphabetic letter are significantly different ($p < 0.05$). Images of human cervical cancer HeLa cells cultured in conditioned mediums (CMs) pre-treated with PBS, LPS, STTP, and LTTP for 0, 24, and 48 h (**B**).

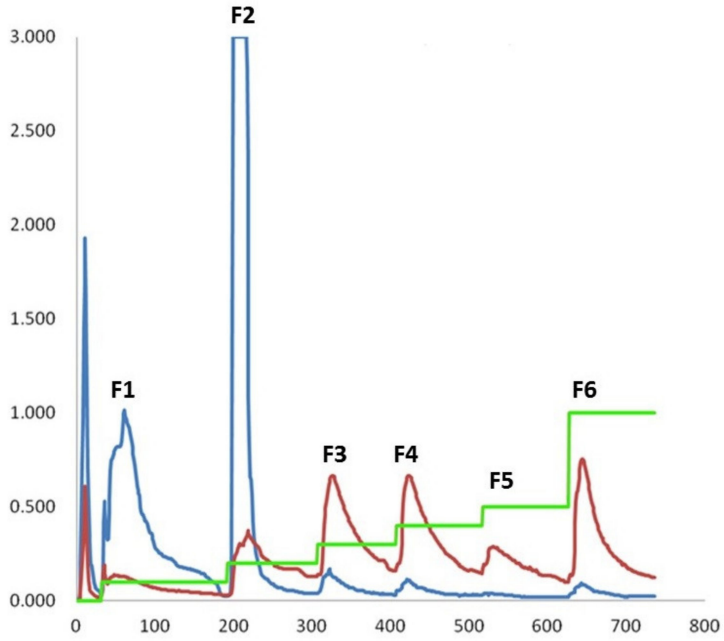


Figure 2. DEAE-sepharose anion-exchange column chromatogram of LTTP. The column was eluted with water, and 0.1–1.0 M NaCl step gradient at a flow rate of 1.0 mL/min. Blue line indicates the amount of polysaccharides in each tube; red line indicates the amount of proteins in each tube; green line indicates the NaCl concentration in step gradient. Numbers of X axis indicate the collecting tube number; values of Y axis indicate the relative concentrations of polysaccharides and proteins, and concentrations of NaCl.

Table 3. Percentages of indicated chemical components of different LTTP fractions.

Sample	Polysaccharides	Triterpenoids	Polyphenols	Proteins	Unknown	pH [a]
F1	12.67	8.67	15.33	0.02	63.31	6.35
F2	21.52	7.33	2.38	0.02	68.75	6.21
F3	25.00	41.33	13.33	0.04	20.30	6.58
F4	5.77	38.08	18.08	0.02	38.05	6.67
F6	0.67	9.56	8.44	0.04	81.29	6.02

[a] Fractions of LTTP were quantified to 200 mL with water before measurement.

3.6. Molecular Weights of Polysaccharides

The pharmacological activity of a polysaccharide largely depends on its structural characteristics, such as Mw, solubility, viscosity, primary structure and advanced structure [28]. For example, the bioactivity of mushroom polysaccharides can be classified into three groups: (a) anti-diabetic activity, with Mw between 3 and 5 kDa; (b) anti-inflammatory activity, with Mw between 10 and 100 kDa; (c) anti-tumor activity, with Mw over 30 kDa [29]. In this study, the Mw of the polysaccharides in each LTTP fraction was determined by HPGFC. Based on calibration with dextran standards, the calculated Mw and percentage of the major polysaccharide in each LTTP fraction were shown in Table 4. The decreasing sequence of Mw was F2 (667 kDa), F1 (279 kDa), F3 (250 kDa), F6 (226 kDa) and F4 (195 kDa). Previously, it is suggested that TTP can be utilized as a potent anti-tumor and immuno-enhancing material in functional food due to the Mw (49.9 kDa) of TTP [8]. According to the previous classifications of mushroom polysaccharides [29], these five LTTP fractions may have anti-tumor activities because all of their major polysaccharides show Mw larger than 30 kDa.

Table 4. The calculated molecular weight (Mw) and percentage of the major polysaccharide in different fractions of LTTP.

Sample	Retention Time	Log Mw	Calculated Mw (kDa)	Percentage (%)
F1	10.51	5.445269	279	83.26
F2	10.27	5.823893	667	80.72
F3	10.54	5.397941	250	93.67
F4	10.61	5.290664	195	72.33
F6	10.57	5.353768	226	90.17

3.7. Effect of Different LTTP Fractions on Growth of Macrophages

We determined whether the five different LTTP fractions affected the growth of RAW264.7 macrophages. The results indicated that the cell viabilities of macrophages treated with the same dose (100 μg/mL) of five different LTTP fractions were F2 > F6 > F3 > F4 > F1, that were 112.68%, 91.67%, 89.80%, 89.57%, and 81.89%, respectively (Figure 3A). NO (nitrite) concentrations in mediums of RAW264.7 macrophages treated with five different LTTP fractions were significantly increased compared to macrophages treated with PBS. The sequence of nitrite concentrations was F1 > F4 > F3 > F6 > F2, that were 45.24 μM, 14.25 μM, 11.35 μM, 5.98 μM and 3.94 μM, respectively (Figure 3B). In addition, the TNF-α concentrations in mediums of RAW264.7 macrophages treated with five different LTTP fractions were significantly increased compared to the PBS group. The sequence of TNF-α concentrations were F1 > F4 > F6 > F3 > F2, that were 6636.62 pg/mL, 6281.32 pg/mL, 6062.84 pg/mL, 5661.08 pg/mL, and 3583.92 pg/mL, respectively (Figure 3C). Our previous study showed that *T. triangulare* extracts inhibited leukemic U937 cell growth due to the abilities to stimulate human monocyte growth and secrete cytokines (IL-1β, IFN-γ, and TNF-α) [7]. Wang et al. showed that RAW264.7 macrophages were activated by TTP to produce NO and increase secretion of cytokines and receptors (iNOS, TLR2, TLR4, and IL-1β) [8]. From these results, it can be concluded that the five different fractions of LTTP extract are not only capable of stimulating RAW264.7 macrophages to produce NO, but also to secrete cytokines. In particular, the survival rate of RAW264.7 macrophages was the highest in F2 fraction, but F1 fraction showed the highest concentrations of NO and cytokines in medium.

3.8. SW620 Cell Survivals Following Cultured with Condition Media of RAW264.7 Macrophages Pre-Treated with Different LTTP Fractions

Previous results showed that both CMs of LTTP- and STTP-treated RAW264.7 macrophages could inhibit human HeLa cell growth (Figure 1B). We next tested whether CMs of RAW264.7 macrophages pretreated with five different LTTP fractions could inhibit cell growth of human colon cancer SW620 cells. RAW264.7 macrophages were treated with the same dose (100 μg/mL) of different LTTP fractions for 48 h to obtain different CMs. The inhibitory effects on the growths of SW620 cells were studied following treatments with different CMs. The experimental results indicated that CMs from pre-treatment with five different LTTP fractions significantly inhibited the growths of SW620 cells when compared to the PBS-pretreated group (Figure 4). The cell viabilities in decreasing order of SW620 cells were F2 > F3 > F6 > F4 > F1, that were 79.9%, 53.0%, 50.0%, 48.5%, and 47.8%, respectively. The inhibitory effects of CMs for SW620 colon cancer cells are reversely correlated with nitrite and TNF-α concentrations in CMs (Figure 3B,C). Since the LTTP fractions in the CMs were not washed out, the inhibitory effects of CMs for SW620 cell growth were in part by LTTP fractions. In brief, the F1-pretreated CMs shows the highest concentrations of both nitrite and TNF-α, and also shows the highest inhibitory effects of SW620 cells.

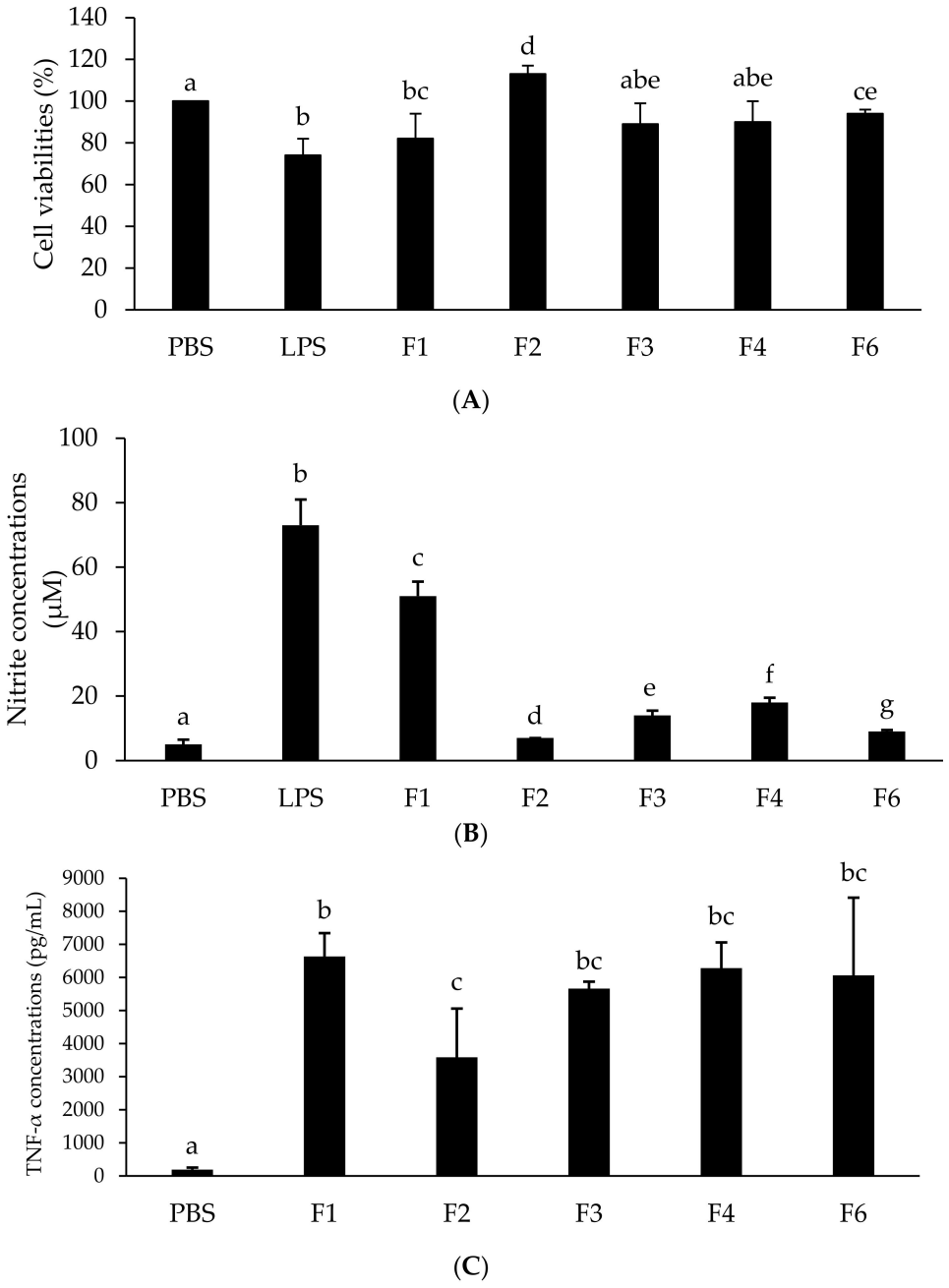


Figure 3. (**A**) Cell viabilities, (**B**) nitrite concentrations and (**C**) TNF-α concentration in mediums of RAW264.7 macrophages treated with PBS, 1 μg/mL LPS (LPS), and the same dose (100 μg/mL) of different fractions of LTTP (F1–F4 and F6) for 24 h. The cell viability using PBS-pretreated CM was designed as 100%. Values (mean ± SD, n = 3 for all test groups) in each column not sharing the same alphabetic letter are significantly different ($p < 0.05$).

3.9. Fourier-Transformed Infrared (FTIR) Spectroscopy Analysis

As a complementary chemical analysis method, FTIR spectra provide a rough identification of polysaccharides present in samples. The F1 and F2 fractions of LTTP showed significant different effects in survivals of RAW264.7 macrophages, inducing NO and TNF-α secretions of macrophages (Figure 3), and inhibitory effects for SW620 cancer cells. The FTIR spectra of F1 and F2 fractions of LTTP were analyzed (Figure 5). The band between 1035^{-1} and 1100^{-1} is associated with the stretching vibrations of COC and COH bonds. The characteristic signals between 1400^{-1} and 1450^{-1} are attributed to the sugar ring and glycosidic bond C-O stretching vibrations. The absorption band at 1640^{-1} is associated with the stretching of C=O bonds [30], whereas the absorption band between

1600^{-1} and 1700^{-1} is representative of a primary amine group [31]. The broad signal at 3300^{-1} to 3400 cm^{-1} is attributed to the stretching vibration of O-H bonds in constituent sugar residues and a signal at 3100^{-1} to 3250 cm^{-1} is attributed to the stretching vibration of N-H groups. The weak absorption band at 3275^{-1} to 3332^{-1} indicates a CH group of a benzene ring segment [32]. Compared to the spectrum of the ursolic acid standard [33], F1 fraction showed stronger but F2 fraction showed weaker absorption bands. In brief, the results of FTIR spectra identified that chemical components of the F1 and F2 fractions of LTTP included chemical structures of triterpenoids, polyphenols, sugars, and proteins as results shown in Table 3.

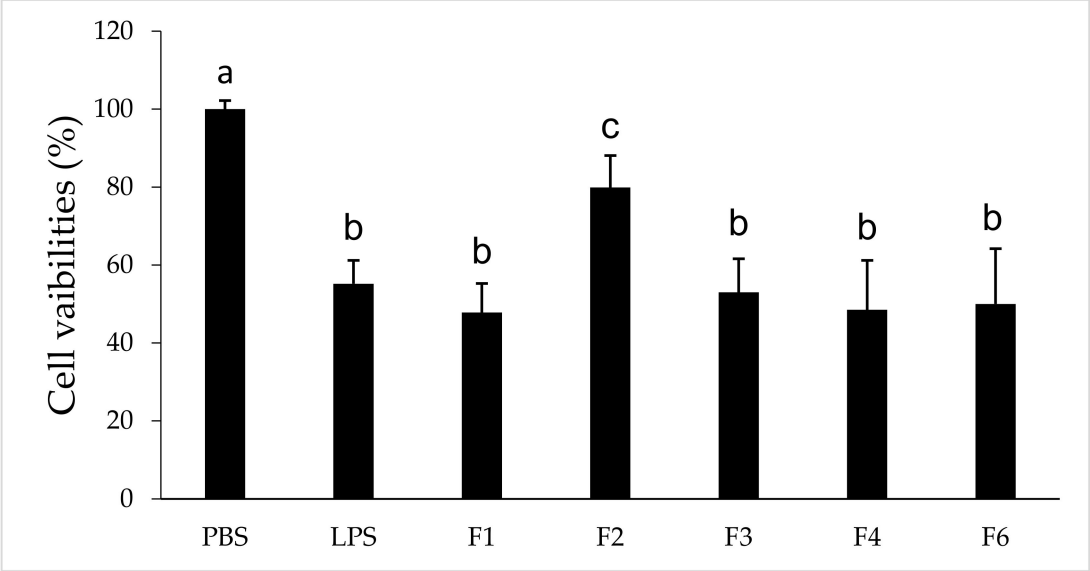
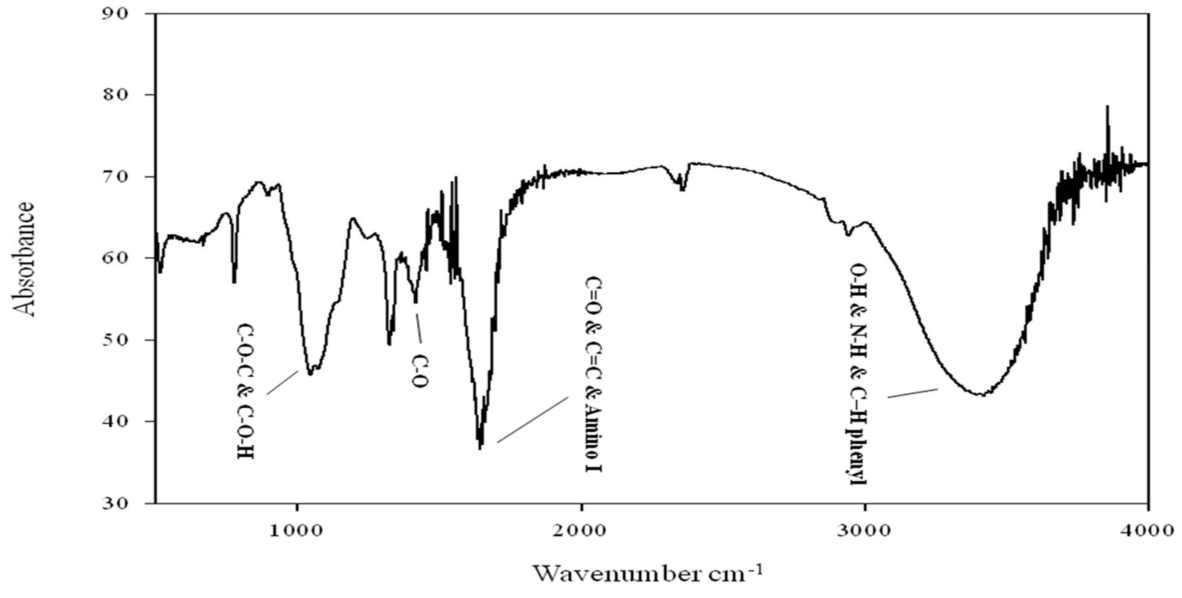
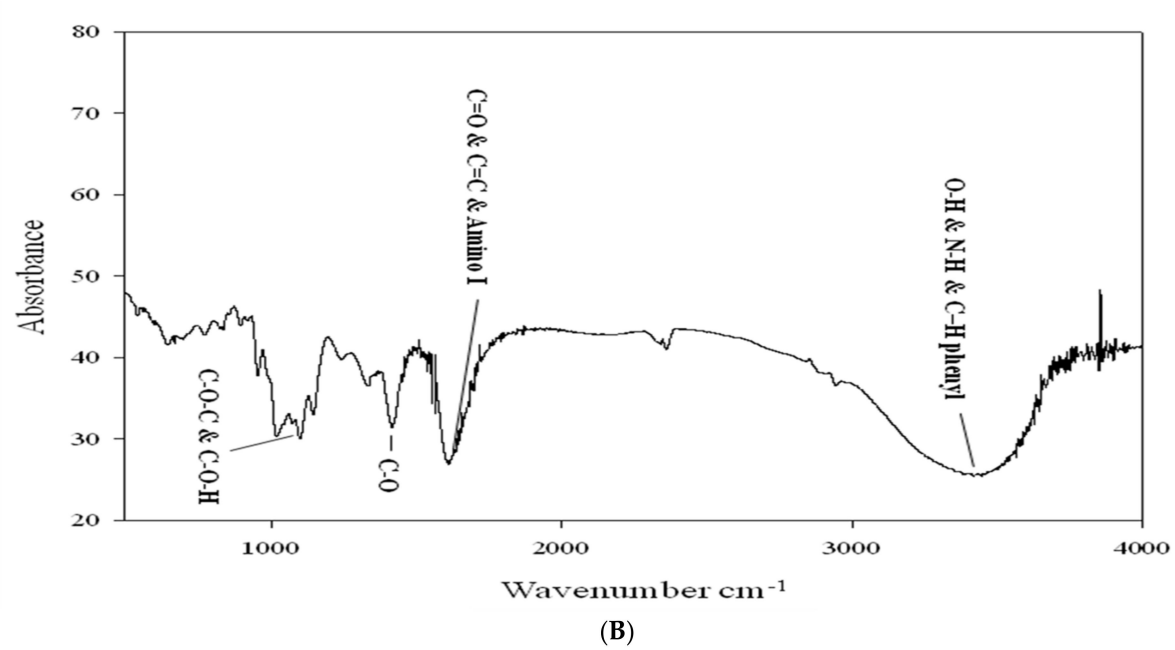


Figure 4. Cell viabilities of human colon cancer SW620 cells cultured in different CMs pretreated with PBS or the same dose (100 μg/mL) of different fractions of LTTP (F1–F4, F6) for 48 h. The cell viability using PBS-pretreated CM was designed as 100%. Values (mean ± SD, n = 3 for all test groups) in each column not sharing the same alphabetic letter are significantly different ($p < 0.05$).



(**A**)

Figure 5. *Cont.*



(**B**)

Figure 5. FTIR spectra of F1 (**A**) and F2 (**B**) fractions of LTTPs.

Preliminary phytochemical studies have reported the presence of phenolic [7,9], betalains [10], carotenoids [34], alkaloids, flavonoids [7,11,35,36], saponins, and tannins in the leaves and leaf extract of *T. triangulare* [37–39]. Moreover, Amorim et al. identified seventeen compounds in the stem and leaves of *T. triangulare*, including four new compounds: one acrylamide and three pheophytins [40]. Our results show similar chemical components in the LTTP. The results of the present study showed that fractions (F1–F4 and F6) of LTTP contained polysaccharides, triterpenoids, polyphenols, and proteins (Table 3), noting that the different types of polysaccharides, such as polysaccharide-proteins and polysaccharide-triterpenoids, are associated with anti-tumor properties. The extracts of *T. triangulare* with greater antioxidant activities show stronger immunomodulatory activities due to these bioactive phytochemicals and polysaccharides.

4. Conclusions

In this study, we confirmed that UAE method is a better than HRE method for extraction of TTP, and the optimal conditions were: temperature 85 °C, ultrasonic power 600 W, extraction solid/liquid ratio 1/20, and extraction time 75 min, for a polysaccharide yield of 3.14%. CMs of RAW264.7 macrophages pretreated with both LTTP and STTP significantly inhibited cervical cancer HeLa cell growths. However, LTTP exhibited better immunomodulatory activity than STTP. Furthermore, six LTTP fractions (F1–F6) were separated using DEAE-Sepharose Fast Flow column and eluted by NaCl step gradients. Excluding F5, the Mw of the five major polysaccharides from different LTTP fractions were all greater than 100 kDa. These 5 fractions of LTTP showed significant amounts and signals of chemical structures of polysaccharides, triterpenoids, polyphenols and proteins. These fractions not only stimulated NO and TNF-α secretions of RAW264.7 macrophages but also inhibited growths of colon cancer SW620 cells. In particular, F2 fraction of LTTP showed the highest immunomodulatory effect among these LTTP fractions. Therefore, it is suggested that LTTP, especially the F1 fraction, can be utilized as a potent anti-tumor and immunomodulatory material in functional food or Chinese medicine.

Author Contributions: Conceptualization, W.-K.H. and M.-S.T.; data curation, G.-G.L. and H.-B.L.; formal analysis, S.-H.Y. and M.-S.T.; investigation, Z.-Q.C. and B.-H.J.; methodology, S.-H.W. and H.-K.T.; resources, C.-W.H. and M.-S.T.; supervision, W.-K.H. and M.-S.T.; validation, S.-H.Y. and M.-S.T.; visualization, Z.-Q.C. and S.-H.W.; writing—original draft, S.-H.Y. and Z.-Q.C.; writing—review and editing, S.-H.W. and M.-S.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded in part by the Ministry of Science and Technology of Taiwan (MOST 109-2221-E-005-031-MY3) and Taichung Veterans General Hospital/Da-Yeh University Joint Research Program (TCVGH-DYU1108302).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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