

## RESEARCH ARTICLE

Pharmaceutical Production of Anti-tumor and Immune-potentiating *Enterococcus faecalis*-2001  $\beta$ -glucans: Enhanced Activity of Macrophage and Lymphocytes in Tumor-implanted Mice

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**Abstract: Background:** *Enterococcus faecalis* 2001 is a probiotic lactic acid bacterium and has been used as a biological response modifier (BRM). From physiological limitation of bacterial preservation in storage and safety, the live *E. faecalis* 2001 has been heat-treated and the BRM components containing high level of  $\beta$ -glucan, named EF-2001, were prepared.

**Method:** The heat-treated EF-2001 has been examined for the antioxidative potential for radical scavenging and anti-tumor activities as well as immune-enhancing response in mice. Lymphocyte versus polymorphonuclear leukocyte ratio was increased in mice upon treatment with EF-2001. The number of lymphocytes was increased in the EF-2001-treated group. In the mice bearing two different Ehrlich solid and Sarcoma-180 carcinomas, the treatment with EF-2001 resulted in anti-tumor action. Tumor-suppressive capacity upon treatment with EF-2001 was significantly increased compared to normal controls.

**Results:** During the time interval administration of 5 weeks between the priming and secondary administration of EF-2001, the expression and production levels of TNF- $\alpha$  were also observed in the EF-2001-administered mice. Additionally, anti-tumor activity examined with the intravenous administration of EF 2001 with a 34 time intervals was also observed, as the growth of Sarcoma180 cells was clearly inhibited by the EF-2001.

**Conclusion:** From the results, it was suggested that the immune response is enhanced due to antioxidative activity caused by the EF-2001 and anti-tumor activity by NK cells and TNF- $\alpha$ .

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## 1. INTRODUCTION

Cancers and immune disorders have historically been regarded as intractable diseases and therefore, there has been great attention to treat and prevent them. Current development of alternative and complementary functional medicines

is the important subject of the biomedical science [1]. Although there has been a considerable progress in the development of antitumor agents and drugs, tumor is regarded as the first death factor through the world [2]. Although tumor and immune susceptibilities are diverse in human population, and therefore, prevention of tumorigenesis and immune disorders is important on the basis of personal genetics and medicines [3]. As alternative approaches, many plant-based candidates have been searched and soybean isoflavones and plant resources have been used for therapeutic approaches to treat and prevent various cancers including breast cancer and

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immune disorders including inflammation [4, 5]. For example, some flavonoids stimulate immune capacity of monocyte/macrophage function and cancer regression with anti-mutagenic potentials in mouse lymphatic cells of the immune system [6-8].

*Enterococcus faecalis* as a lactic acid bacterium was separated from the commensal microbiota of human. The lactic acid bacteria are recently receiving great attentions associated with functionality [9, 10]. For example, a lactic acid bacterium has recently been evaluated as a potential hospital opportunistic anti-fungal agent [11]. In addition, multi drug-resistant *E. faecalis* strains have been reported to raise the bacteremia-causing issue in the patients with immunocompromise [12]. Therefore, *E. faecalis* has been decorated as a source of human consumption from the international authorities such as FDA, USA. In addition, *E. faecalis* strain has been studied for the food industry and pharmaceutical applications and *E. faecalis*-based probiotics has been developed for use in food. Recently, *E. faecalis* has been used for functionality and medicinal food resources as meals of normal human beings [13, 14]. A few years ago, our group established that *E. faecalis* 2001 (EF2001) releases a single component of natural  $\beta$ -glucan as a non-estrogen probiotic [15]. *E. faecalis* 2001 has been shown to exhibit plant estrogen-like activity. In addition, *E. faecalis* 2001 inhibits the tyrosine kinase and DNA topoisomerase II activities with the host's immune capacity. In addition, anti-tumor and analgesic effects of the active fraction have also been observed in the *E. faecalis* 2001. [15]. In patients with atherosclerosis and osteoporosis, the beneficial effects were observed in the relevance of increased attention of the degeneracy of the breast cancer and prostate cancer [16]. With respect to radiation protection of  $\beta$ -glucans, it was reported to have a delay in death and tumor growth inhibitory capacity in tumor-bearing mice in the whole-body X-ray-irradiated mice when injected intraperitoneally [17]. Mice treated with  $\beta$ -glucans showed the increased levels of white blood cells and lymphocytes, indicating that antitumor effects of  $\beta$ -glucan are based on activated hematopoietic and enhanced anti-tumor immunity [17]. In a similar study,  $\beta$ -glucans of *Agaricus blazei* Murill have also been suggested to have a tumoricidal activity at the time of oral administration in syngeneic and heterogeneous tumor-bearing mice [16, 18]. When the mice were administered with the water soluble  $\beta$ -glucans of *A. blazei* Murill, type I cytokine IFN- $\gamma$  and anti-tumor agent TNF- $\alpha$  are mainly expressed in the peritoneal innate immune cells. The  $\beta$ -glucans have also been shown to have the anti-oxidant activity in cell culture systems and animals.

In the present study, as an anti-tumor and immune-potentiating agent, the EF-2001 has been prepared and formulated from *E. faecalis* 2001, the heat-treated probiotic lactic acid bacterium as a biological response modifier (BRM). To obtain the preserved bacterial sources with storage, safety and stabilities, the live *E. faecalis* 2001 has been thoroughly heat-treated. For the active  $\beta$ -glucan-enriched preparation, fractionation process with edible solvents has been performed and the EF-2001 has been designated and commercially supplied. The prepared EF-2001 has been examined for the anti-tumor activity and immune-enhancing responses in the experimental model mice. For the immune activation of the EF-2001, lymphocyte versus polymor-

phonuclear leukocyte ratio was evaluated. In addition, for the direct *in vivo* anti-tumor activity, the 2 different Ehrlich solid and Sarcoma-180 carcinoma cells were used to check the TNF- $\alpha$  production in the EF-2001-administrated mice. From the results, it was evident that EF-2001 activated the immune system such as macrophages, lymphocytes and NK cells, and suppressed the tumor growth by induction of endogenous TNF- $\alpha$ . Therefore, it is suggested that EF-2001 is a promising sub-functional agent for cancer patients.

## 2. MATERIAL AND METHODS

### 2.1. Regents

Unsaturated fatty acid, linoleic acid, has been supplied from Nacalai Tesque, Inc (Tokyo, Japan). Several inorganic reagents such as  $\text{NH}_4\text{CNS}$  (ammonium thiocyanate), ferrous (iron II) chloride tetrahydrate, HCl (hydrogen chloride) solution,  $\text{C}_2\text{H}_5\text{OH}$  (ethanol),  $\text{K}_2\text{HPO}_4$  (potassium phosphate dibasic, anhydrous, grade ACS Product Code PB0447, Merck) and  $\text{KH}_2\text{PO}_4$  (potassium phosphate monobasic) were purchased from Merck & Co., Inc (Kenilworth, NJ, USA). Anti-oxidation and buffer reagents of 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2-N-morpholino ethane-sulfonic acid, hydrate (MES) were purchased from Merck & Co., Inc (Kenilworth, NJ, USA). Positive trolox as a standard DPPH substance of 400  $\mu\text{M}$  was purchased from (Aldrich Chemical Company, CA, USA). Chromogenic substrate 3,3',5,5'-tetramethylbenzidine (TMB) has been supplied from Thermo Fisher Scientific Inc. (MA, USA). The reagents were of commercially available pharmacological grades.

### 2.2. Biotechnological Production of *Enterococcus faecalis* 2001

The body of the bacteria that has been heat-treated is free of fungal product (*Enterococcus faecalis* 2001) was named EF-2001. Commercially applicable *Bacillus* products, EF-2001<sup>®</sup> heat-treatment extract, have, therefore, been constructed from dextrin and gelatin. For routine research, EF-2001<sup>®</sup> is obtained from the stock of Nippon BRM Co., Ltd. (Tokyo, Japan).  $\beta$ -1,3- Glucan as a main component was present in gram rate of 6.5 mg/EF-2001<sup>®</sup>. "EF-2001" and " $\beta$ -glucan" are subsequently used as synonyms. EF-2001 was suspended in physiological saline for further study of 2,4,8% strength (w/v).

### 2.3. Animals

Male ICR mice, commercial mice that have been purchased from Japan Clea Inc. were used in a cautious trading and 4 weeks of age. After one week of preliminary breeding, they were on ad libitum diet as an experimentally designed nutrition for controls or experiments. The animals,  $23 \pm 1.0^\circ\text{C}$ , were kept in the experimental house for model animals at  $55 \pm 5\%$  humidity, with 12-hour light-dark cycle. Animal food (CLEA Japan (Ltd.), CE-2) with access to free water was given.

### 2.4. Examination of Scavenging Capacity Using DPPH as a Radical Source

In order to measure the scavenging capacity of DPPH radical, the stable free radicals which have been produced as



the reaction products were measured. Using positive trolox as a standard DPPH substance of 400  $\mu$ M, MES buffer solution of 0.2 M and 80% ethanol, 0.2 mM Trolox and 20% ethanol were prepared before the experiment. A solution of 20 % ethanol (15 ml) and 0.2 M MES buffer solution (pH 6.0) was mixed with 400  $\mu$ M DPPH to prepare 15 ml of mixtures. The mixture 0.9 ml was added to 80% ethanol (300  $\mu$ l). To prepare the test sample, each 0.20% of the EF-2001 prepared was added to the mixture of 0, 30, 60, 90, 120 and 150  $\mu$ l at the time intervals of each 30 consecutive second. After the solution was loaded to a vortex apparatus, the samples were continuously stirred. The samples were then measured with 520 nm absorbance using a spectrophotometer at the time intervals of 0.5 and 20 minutes. Trolox with a water-soluble derivative of positive standard substance and Tocopherol, adjusted to 0.2 mM concentration, were added to the serially increasing concentrations of 0, 30, 60, 90, 120 and 150  $\mu$ l.

### 2.5. Determination of the Anti-oxidative Potential of EF2001 $\beta$ -glucan

The anti-oxidative capacity of EF2001  $\beta$ -glucan was examined using the Rhodan iron method in order to evaluate the levels of lipid peroxides produced during oxidation process of unsaturated lipids. Divalent ferrous iron  $\text{Fe}^{2+}$  was oxidized to trivalent ferric iron  $\text{Fe}^{3+}$  when  $\text{Fe}^{2+}$  iron and lipid peroxide (LOOH) reacted. The consequently generated product, red colored  $\text{Fe}(\text{SCN})_3$  is known as the Rhodan iron, when  $\text{Fe}^{3+}$  and ammonium thiocyanate (SCN) further reacted. Peroxidation levels of lipid were then measured and evaluated by colorimetric analysis using a spectrophotometer. When 0.08413 g of linoleic acid was dissolved with 3 ml of ethanol in a test tube, the dissolved linoleic acid solution was further diluted to the final volume of 15 ml of 0.1 M sodium phosphate buffer to adjust to 0.02 M sodium phosphate buffer. The solution was subsequently used for the next experiments. For the measurement of the capacity, 0.4 ml of 0.1 M sodium phosphate buffer and 0.1 ml of 1% EF-2001 were added to the solution of 0.5 ml of 0.2 M linoleic acid, and used as a test sample. Lipid auto-oxidation using the linoleic acid progressed by constant stirring at 37°C in a time-dependent manner for 1, 3, 5 and 6 days. As a positive control, Trolox was used, while distilled water for the negative control. A solution containing 0.05 ml of 0.2 mM  $\text{FeCl}_2$  as  $\text{Fe}^{2+}$  form in 3.5 % HCl, 2.35 ml of 75 % ethanol and 0.05 ml of 30 %  $\text{CH}_4\text{N}_2\text{S}$  reagent (Rhodan ammonium) was added to the above reaction mixture after the time intervals of 24, 72, 120 and 267 hours. After 3 min, absorbance at 500 nm was subsequently observed using a spectrophotometer [19, 20].

### 2.6. Evaluation of Immune Cells by Ratio of Lymphatic Cells Versus Polymorphonuclear Leukocytic Cells

The evaluation method described by our previous report has been applied for the measurement of L/P ratio in mice [17,18]. Twenty litters of neonatal Swiss-Webster mice (Japan SLC, Inc., Tokyo, Japan) (6-12 hr after birth), which are known to be premature, were grouped to two different experimental teams, each having 10 litters. A group as a control was subjected to intraperitoneal (i.p.) injection of 0.02 ml of saline, whereas in the EF-2001 sample-administrating groups, each EF-2001 (200  $\mu$ g/mouse) in 0.02 ml solution

fluid was intraperitoneally injected into the mice. To obtain blood samples, the tail vein of each mouse was subjected to the blood collection before i.p. injection and 1, 3, 6, 10, and 14 days after i.p. injection of the control group and EF-2001-treated group. To count the cells, Wright's cell staining was applied on the thin-layer blood smears prepared, as previously reported for the thin-layer blood smears and Wright's cell staining [18]. Then, total cell numbers containing 100 lymphocytes and polymorphonuclear leukocytes were counted using a light microscope in a general contrast. The ratio of the number of lymphatic cells versus that of polymorphonuclear leukocytes was calculated as the L/P ratio. Results obtained for the lymphocyte versus polymorphonuclear leukocyte ratio were calculated and evaluated, as previously described by Gu [17]. A Student's *t*-test was applied for determining the statistical significance with a *p* value <0.05.

### 2.7. Evaluation of Anti-tumor Capacity in Ehrlich Carcinoma-bearing Mice

Each group having 10 male 4-week-old ICR mice (Japan SLC, Co., Tokyo, Japan) was subjected to the anti-tumor activity using Ehrlich solid carcinoma (ESC). Twenty-four hours later, after inoculation and transplantation of Ehrlich solid carcinoma cells ( $1 \times 10^6$  cells, 0.02 ml) or pure water (1.0 ml/100 g body weight), with EF-2001 (200 mg/kg body weight), each mouse was subjected to the oral administration for 35 days in the same condition. The tumor volume or size was assessed at different intervals and five times for 35 days by measuring changes in tumor volume, as expressed by length x width x thickness ( $\text{mm}^3$ ). Tumor tissues were removed and isolated 35 days after inoculation to measure the tumor weight (g). The inhibition ratio (IR) of tumor growth (inhibition of the growth of the EF-2001-treated group vs. control group) was calculated from the animal experiments.

### 2.8. Evaluation of Anti-tumor Capacity in Sarcoma 180 Carcinoma-bearing Mice

Ten male 4-week-old mice of the ICR strain were grouped for inoculation of Sarcoma 180 solid carcinoma cells (Japan SLC, Co., Tokyo, Japan). Sarcoma 180 solid carcinoma cells ( $1 \times 10^6$  cells, 0.02 ml) were subcutaneously inoculated into the right inguinal area of the experimental mice. Twenty-four hours after inoculation of Sarcoma 180 carcinoma cells, the control group received the pure water (1.0 ml/100 g B.W.) by oral administration and the experimental group was subjected to the oral administration of the EF-2001 (20.0 mg/100 g B.W.) for 35 days. The tumor volume or size was examined five times during 35 days after inoculation of Sarcoma 180 solid carcinoma cells, as measured for the Ehrlich carcinoma cells. The length of the largest diameter of the tumor was measured. At 35 days after inoculation, tumor tissues were isolated to measure the tumor weight (g), as described above. The IR level of the EF-2001-treatment group was also calculated, as described above and compared with the control group.

### 2.9. Measurement of Interferon- $\gamma$ (IFN- $\gamma$ ) Produced

To uncover the mechanism(s) by which EF-2001 positively modulates the immune system of tumor-bearing mice,



an enzyme-linked immunosorbent assay (ELISA) was applied to measure the produced IFN- $\gamma$  levels using an IFN- $\gamma$ -detection kit, which was specific for the mouse-derived IFN- $\gamma$  (Amersham Biosciences, Inc.). Each group of 10 male 4-week-old mice as the ICR type was used supplied from Japan SLC, Co. (Tokyo, Japan). Sarcoma-180 carcinoma cells ( $1 \times 10^6$  cells) cultured *in vitro* were subcutaneously inoculated into the right inguinal area of each mouse. The control group received pure water (1.0 ml/100 g B.W.) by oral administration during the experimental period, while the treatment group received the EF-2001 (20.0 mg/100 g B.W.) by oral administration. After 4 weeks of the EF-2001 administration, each 5 ml of PBS buffer was intraperitoneally injected into each mouse and the abdominal regions were rubbed, and peritoneal fluids were collected. The peritoneal fluids were then kept in a freezer at  $-20^\circ\text{C}$  for 5 hr. After the tissue-derived fluids were defrosted and thawed, the supernatants were subjected to centrifugation (3000 rpm, 10 min). The supernatants were then subjected to dialysis using dialysis membrane and were concentrated using polyethylene glycol-4000 (PEG 4000). From the concentrated samples, each 50  $\mu\text{l}$  of the sample or the standard was added to each well of a 96-well plate and the plates were covered and kept at room temperature ( $25^\circ\text{C}$ ) for 120 min. Thereafter, 50  $\mu\text{l}$  of anti-mouse antibody, which was previously biotinylated, was added and incubated at room temperature ( $25^\circ\text{C}$ ) for further 60 min. The plates were then washed three times with washing buffer. One hundred  $\mu\text{l}$  of streptavidin-conjugated horseradish peroxidase (HRP) solution was mixed with the washed plates for antigen-antibody interaction and incubated at room temperature for 30 min. The plates were washed three times with washing buffer solution. In order to detect the positive interaction, the plates were incubated with 100  $\mu\text{l}$  of TMB solution for 30 min and 100  $\mu\text{l}$  of termination reagent was further added. The yellow-colored products were measured using a Labsystems Multiskan MS-UV spectrophotometer (Dainippon Pharmaceutical Co., Ltd, Tokyo, Japan) at an absorbance of 450 nm within 30 min. The level of IFN- $\gamma$  expression was calculated using a calibration standard.

#### 2.10. Examination of Tumor Necrosis Factor- $\alpha$ (TNF- $\alpha$ ) Level

Examination of TNF- $\alpha$  level, which was expressed in mice, was assessed by an ELISA kit using an anti-TNF- $\alpha$  detection kit that was specific for mouse TNF- $\alpha$  (Calbiochem, Inc. and Merck & Co., Inc.) (Kenilworth, NJ, USA). As described above, ten male 4-week-old ICR mice were used in each group and Sarcoma-180 solid carcinoma cells cultured *in vitro* were subcutaneously inoculated into the right inguinal area of the mice. For the control group, pure water was orally administered (1.0 ml/100 g B.W.), while the experimental EF-2001 groups received the EF-2001 (20.0 mg/100 g B.W.) by oral administration. After four weeks of oral administration, the mice received 5 ml of PBS by intraperitoneal injection. To analyze the fluidal cytokine, the mice abdominal areas were rubbed and peritoneal fluids were collectively prepared. The fluids were then kept in a freezer at  $-20^\circ\text{C}$  for 3 hr and the frozen fluids were defrosted and thawed. The supernatant was consequently collected by centrifugation (3000 rpm, 15 min) and dialyzed, as described

above. The supernatant was concentrated with PEG 4000 and utilized for the experiments. In a 96-well plate, 50  $\mu\text{l}$  of the prepared samples or the standard controls was added to each well of a 96-well plate and the well plates covered were kept to incubate at room temperature ( $25^\circ\text{C}$ ) for 120 min. With 50  $\mu\text{l}$  of biotinyl antibody-containing solution, the wells were further incubated at  $25^\circ\text{C}$  for 2 hr and the plates were washed five times with washing buffer. To develop, each 100  $\mu\text{l}$  of HRP solution was added to the plate covered and incubated at room temperature ( $25^\circ\text{C}$ ) for 30 min. The plates washed five times with washing buffer were incubated with 100  $\mu\text{l}$  of TMB solution for 30 min. During the incubation, the time was decided depending on the intensity of blue color. Finally, to terminate the development, 100  $\mu\text{l}$  of stop buffer solution was added to each plate well and spectrophotometric measurement was carried out in an absorbance at 450 nm using a MS-UV spectrophotometer, as described above. The expressed levels of TNF- $\alpha$  were calculated using a normalized calibration curve.

#### 2.11. Measurement of IgM Levels in Mice

Mouse-specific IgM detection kit has been used and the detecting ELISA assay kit was supplied from BETHYL Laboratories, Inc. (Montgomery, TX, USA) and IgM was purchased as the ELISA starter package. 10 male ICR mice, 4 weeks old, were divided in different groups. The sarcoma-180 solid cancer cells ( $1 \times 10^6$  cells) were injected subcutaneously in the right groin. Control group receiving distilled water was orally administered with 1.0 ml / kg of B.W and sample group was administered with EF-2001 B.W. of 200 mg/kg. 4 Weeks after the administration, blood samples from the retina were collected. After four weeks of administration, bloods samples were also prepared from the fundus. The prepared sera were used by appropriate dilution to prepare 100 times with dilution buffer. Specific antibody was the affinity-purified antibody transferred to 100  $\mu\text{l}$  of the solution into each well of a 96-well plate and incubated for 60 minutes. The specific antibody aspirated was washed well twice with cleaning solution. Then, after the addition of post-coat solution of 200  $\mu\text{l}$  to each well, it was incubated for 30 minutes. A solution of the post-coat was then re-aspirated and cleaned twice with a washing solution. 500-fold was prepared by diluting the sample 100  $\mu\text{l}$  with standard diluent and transferred to each well to dilute the mouse reference sera, incubated for 60 min. Sample/conjugate diluent wells, washed 4 times in a conjugate diluent, were prepared using the goat anti-mouse IgM-Fc in HRP12. Then, 100  $\mu\text{l}$  of the washed antibody/HRP conjugate was added to each well and incubated for 60 min. After washing the wells 3 times with a washing buffer, 100  $\mu\text{l}$  of the TMB substrate was incubated at each well for 10 min. After incubation, the reaction was stopped by adding 2 M  $\text{H}_2\text{SO}_4$  of 100  $\mu\text{l}$  to each well, and was measured using a Labsystems absorbance at 405 nm using a multiscan WS-UV (Dainippon Pharmaceutical Ltd.).

#### 2.12. Measurement of IgG Levels

The levels of IgG produced were examined by a mouse IgG-specific kit (Bethyl Laboratories, Inc., TX, USA) and ELISA kit. Ten male 4-week-old ICR mice were used in each experimental group. Sarcoma-180 solid carcinoma cells

( $1 \times 10^6$  cells) were subcutaneously transplanted into the right inguinal region. As the control group, mice group received pure water (1.0 ml/100 g B.W.) by oral administration and the EF-2001 treatment group received the EF-2001 (20.0 mg/100 g B.W.) by oral administration. Blood samples were prepared from the eyeground of mice after 4 weeks after administration and sera were used for the detection of IgG levels. As described above, the specific capture antibody was prepared by dilution of affinity-purified antibody for 100-fold with the coating solution and 100  $\mu$ l of the resultant solution was added to each well and incubated for 60 min. The specific capture antibody in wells was removed by aspiration and wells were washed. Each well was added with 200  $\mu$ l of post-coated solution for 30 min and post-coated solution was also removed, following washing. To examine the IgG levels, each 100  $\mu$ l of the EF-2001-treated sample and the control were added to each well and incubated for 60 min, followed by four times washing. Antibody/HRP conjugates were constructed by dilution with goat anti-mouse IgG-Fc-HRP conjugate. One hundred  $\mu$ l of antibody/HRP conjugates were added to each well with controls for 60 min, followed by washing for four times. As a termination reagent, TMB substrate (100  $\mu$ l) was transferred to the wells and the wells were incubated for 7 min. By stopping the reaction with 100  $\mu$ l of sulfate solution (2M  $H_2SO_4$ ), absorbance at 450 nm was examined using a Lab system Multiskan MS-UV spectrophotometer.

### 3. RESULTS

#### 3.1. Antioxidative Potential of EF-2001 as Assessed by DPPH Radical Scavenging Activity

EF-2001 treatment resulted in an increase in radical scavenging capacity when DPPH was used. In a control experiment, the water showed no scavenging activity of radicals. Interestingly, the scavenging activity of EF-2001 was much greater than that of a well-known, positive control, 0.2 mM Trolox as shown in Fig. (1).

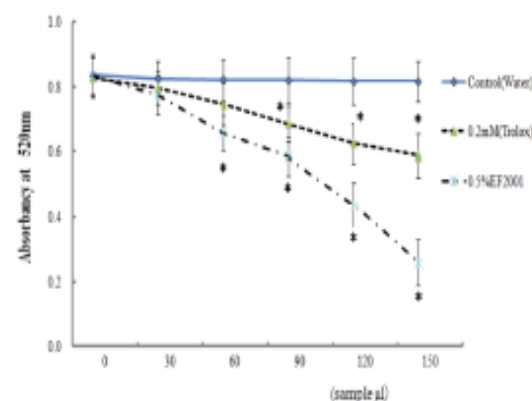


Fig. (1). DPPH radical scavenging-activity of EF-2001. EF-2001 treatment showed higher radical scavenging activity than that of 0.2 mM Trolox, a positive reagent. \* indicates a significantly different ( $p < 0.01$ ) from the control group.

#### 3.2. Antioxidative Potential of EF-2001 as Measured by the Rhodan Iron Method

EF-2001 administration was measured for its ability to inhibit auto-oxidation using linoleic acid, an unsaturated fatty acid. EF-2001 treatment resulted in maximal inhibitory values at day 3; thereafter, the inhibition of auto-oxidation tended to decrease. The positive control, 0.2 mM Trolox resulted in little or no increase in the inhibition of auto-oxidation as compared to 0.5% EF-2001 (Fig. 2).

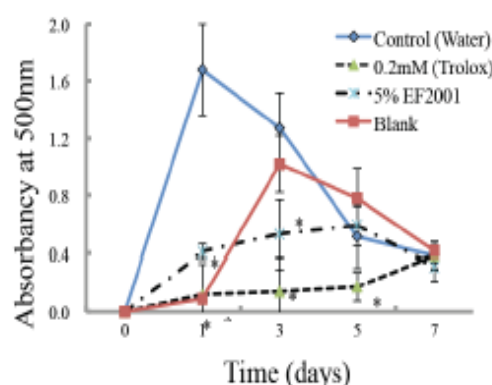


Fig. (2). Anti-oxidative activity of EF-2001. Antioxidant activity was assessed by measuring the auto-oxidation level of unsaturated fatty acid, linoleic acid. Control indicates the maximum peroxide value in the first day. It was then decreased. As described in Materials and Methods, autooxidation of linoleic acid was measured using the ferric thiocyanate method. \* indicates a significantly different ( $p < 0.01$ ) from the control group.

#### 3.3. Increase in Lymphocyte Versus Polymorphonuclear Leukocyte Ratio (L/P ratio) Following EF-2001 Treatment

In order to measure the lymphocyte versus polymorphonuclear leukocyte ratio (L/P) of EF-2001, 10 neonatal Swiss-Webster mice were divided into the two different EF-2001 and control groups. Saline was i.p injected into the control group, and EF-2001, dosing 200  $\mu$ g/mouse, was i.p injected into the experimental group. The arrow (†) indicates the injection day, as shown in Fig. (3). Significant differences in L/P ratio between the control group and the EF-2001-treated group ( $p < 0.01$ ) were observed on days 6, 10, and 14 of post-administration. The L/P ratios of the mice given EF-2001 (200  $\mu$ g/mouse) were gradually increased, depending on the administration days. The L/P ratios in mice given EF-2001 were significantly greater ( $p < 0.01$ ) than those of the control group.

#### 3.4. Anti-tumor activity of EF-2001 on Ehrlich solid Carcinoma

In mice bearing Ehrlich solid carcinomas, 34 consecutive days of oral EF-2001 administration (200 mg/kg B.W.) resulted in 83.1 % suppression in tumor size ( $p < 0.01$ ), as shown in Table 1 and Fig. (4).



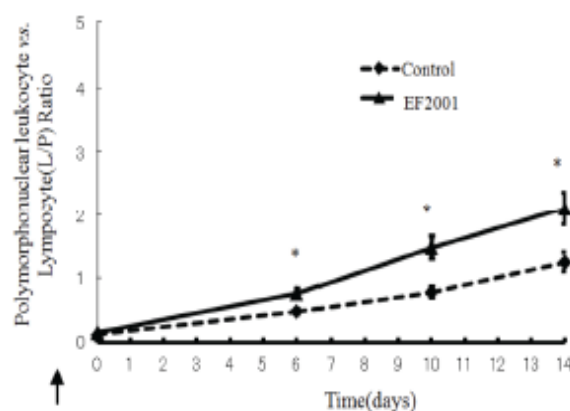


Fig. (3). EF-2001-modulated increase in lymphocyte versus polymorphonuclear leukocyte ratio (L/P ratio). Blood was collected from the tail veins of mice. Blood smears were stained with Wright stain. 100 total lymphocytes and polymorphonuclear leukocytes were counted in order to assess the ratio of lymphocytes to polymorphonuclear leukocytes (L/P ratio). The L/P ratio was assessed each day after intraperitoneal administration of EF-2001 (200 µg/mouse) in neonatal Swiss-Webster mice. The arrow (↑) shows the injection day. Significant differences between the control group and the EF-2001 group were found on 6, 10, and 14 days post-injection. \* indicates a significantly different ( $p < 0.01$ ) from the control group (saline).

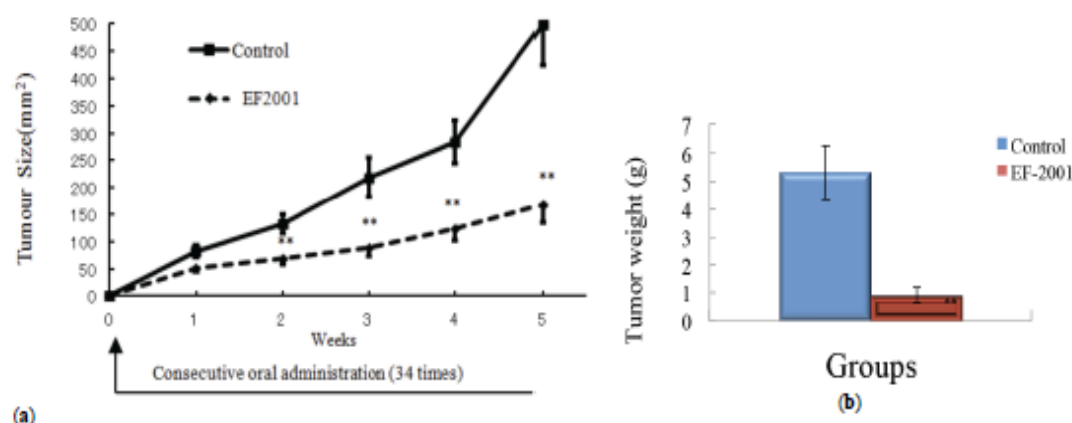


Fig. (4). Tumor growth-inhibitory effect of EF-2001 in Ehrlich cancer cells-bearing mice. Changes in the size of the tumor (A). Changes in the tumor weight (B). After tumor implantation, we measured the weight of the fifth week of the tumor of each group. The results represent  $\pm$  a S.D. \* significantly different from the control group ( $p < 0.05$ ). \*\* Significantly different from the control group ( $p < 0.01$ ).

Table 1. Antitumor effect of the orally administered EF-2001 on the growth of Ehrlich solid carcinoma cells in mice.

Sample	Dose (mg/kg B.W. × Days PO)	Administration Days	Number of Mice	Tumor Size (mm <sup>3</sup> )	Tumor Weight (g)	Inhibition Rate (%)
Control	10 × 34	1-34	10	491.8 ± 77.9	5.31 ± 0.98	-
EF-2001	200 × 34	1-34	10	127.6 ± 25.6**	0.90 ± 0.28**	83.1**

\*\* Statistically significant ( $P < 0.01$ ) from the control group.

### 3.5. Anti-tumor Activity of EF-2001 on Sarcoma 180 Solid Carcinoma

In mice bearing Sarcoma 180 solid carcinomas, 34 consecutive days of oral EF-2001 administration (200 mg/kg B.W.) resulted in a 0.3% suppression in tumor size ( $p < 0.05$ ), as shown in Table 2 and Fig. (5).

### 3.6. IFN- $\gamma$ -Producing Activity of EF2001 in Mice Bearing Sarcoma 180 Tumors

For the detection of IFN- $\gamma$ , a mouse IFN- $\gamma$  ELISA kit was used. As the results are shown in Fig. (6A), the *in vivo* production of IFN- $\gamma$  in EF-2001-administered Sarcoma180 car-

Table 2. Antitumor effect of orally administered EF-2001 on the growth of Sarcoma180 carcinoma cells in mice.

Sample	Dose (mg/kg B.W. $\times$ Days PO)	Administration Days	Number of Mice	Tumor Size (mm <sup>3</sup> )	Tumor Weight (g)	Inhibition Rate (%)
Control	10 $\times$ 34	1-34	10	603.7 $\pm$ 85.2	7.05 $\pm$ 1.57	-
EF-2001	200 $\times$ 34	1-34	9	346.2 $\pm$ 78.3**	2.80 $\pm$ 1.13 *	60.27 *

\* Statistically significant ( $P < 0.05$ ) from the control group.

cinoma-carrying mice was significantly increased when compared to the control group ( $P < 0.05$ ) (Fig. 6A).

### 3.7. TNF- $\alpha$ -Producing Activity of EF-2001 in Mice Bearing Sarcoma 180 Tumors

TNF- $\alpha$  is well known to bring about tumor cytotoxicity in tumor-bearing animals. Therefore, TNF- $\alpha$  production was examined following EF-2001 administration in mice bearing Sarcoma180 tumors. The mouse TNF- $\alpha$  ELISA kit (Pierce Biotechnology) was used to measure TNF- $\alpha$  (Fig. 6B). Simi-

lar to IFN- $\gamma$  production, the mice with the administration of EF-2001 significantly elevated the production of TNF- $\alpha$ .

### 3.8. IgM-producing Activity of EF-2001 in Mice Bearing Sarcoma180 Tumors

The immunoglobulin-producing capacity of B cells is based on the cells when they interact with antigens. Initially IgM class antibodies are expressed on the surface of B cells and functions as receptors; they are regarded as an indication of an immunologically reactive immune system. Therefore, we have examined the effect of EF-2001 to IgM production in mice with sarcoma 180 tumor. Mouse IgM assay kit and ELISA starter accessories package were used to measure IgM. As Fig. (6C) shows, the production of IgM is dramatically increased following EF-2001 administration ( $P < 0.01$ ).

### 3.9. IgG-producing Activity of EF-2001 in Mice Bearing Sarcoma180 Tumors

Because antigen-activated B cells are converted into IgG-producing plasma B cells, in the Sarcoma 180 tumor-bearing mice, we examined the effect of EF-2001 on the production of IgG. A mouse IgG ELISA quantitation kit was used to measure IgG; the results are shown in Fig. (6D). EF-2001 treatment significantly increased IgG levels in the mice bearing Sarcoma 180 tumors, as compared with control mice ( $P < 0.01$ ).

## CONCLUSION

From the results of this study, EF-2001 having a high concentration of  $\beta$ -glucan stimulated the immune system in mice and exhibited antioxidant and anti-tumor activity [21]. It was previously reported that *Lactobacillus* supplementation in protecting hamster lipid peroxidation in serum and liver reduced the level of low density lipoprotein [22]. Inhibition of oxidation events in the internal organs and hairless mice induced by ultraviolet light in the skin has also been observed [23].

With respect to the main component of the EF-200, there is also a possibility that  $\beta$ -glucan is not directly responsible for its anti-oxidant activity, but it brings a high antioxidant activity for the direct destruction of the tumor cells. Since lymphocytes specifically recognize the pathogenic bacteria to induce an adaptive immune response, the mechanism of action of EF-2001 is to be specifically investigated. Therefore, the ratio of lymphocytes versus polymorphonuclear leukocytes was measured as a measure of immune enhancing capacity [24]. EF-2001 increased the L/P ratio in the *in vivo* lymphocyte count and the increased amount was significant in the mice group treated with EF-2001.

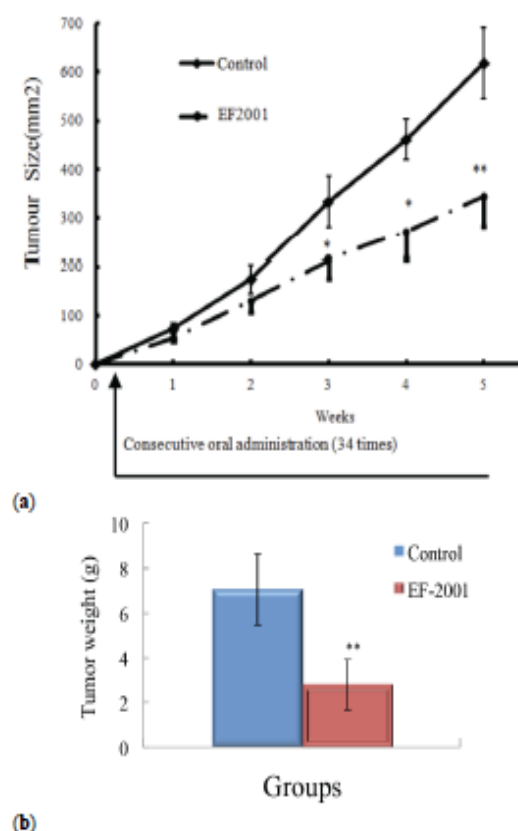


Fig. (5). EF-2001 effect of on tumor growth in mice bearing sarcoma 180 cancer. Change in the size of the tumor (A). Changes in tumor weight (B). Mice were subjected to each process. After tumor implantation, we measured the weight of the fifth week of the tumor of each group. The result means represents  $\pm$  a S.D. \* control group ( $P < 0.05$ ) and significantly different. \*\* From the control group ( $P < 0.01$ ) and significantly different.

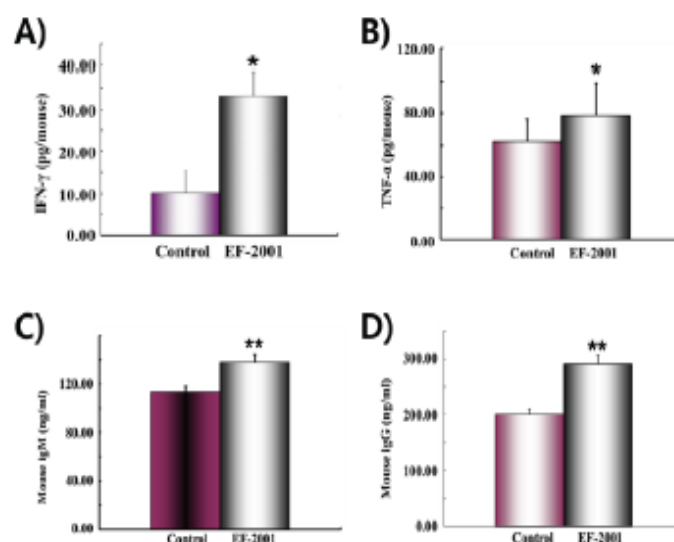


Fig. (6). The effects of the EF-2001 of repeated administration on the levels of IFN- $\gamma$  (A), TNF- $\alpha$  (B), IgM (C) and IgG (D) in mice with Sarcoma180 cancer. Changes in the levels were obtained. The results measured represent  $\pm$  S.D. IFN- $\gamma$  and TNF- $\alpha$  concentration (pg / mouse) as well as IgM and IgG concentration (ng / ml) were also measured (\*  $P < 0.05$ ), \*\* significantly different from the control group ( $p < 0.01$ ).

The *in vivo* antitumor mechanism of EF-2001 in Sarcoma 180 solid and Ehrlich solid carcinoma-bearing mice is interesting. To uncover the EF-2001-induced immune response in tumor-bearing mice, we examined the effects of the EF-2001 on inflammatory cytokine production. As shown in Fig. (6), IFN- $\gamma$  levels were increased in mice bearing Sarcoma 180 solid tumors after administration of EF-2001. Cellular immune activation is suggested to be indeed activated by EF-2001, because IFN- $\gamma$  is exclusively produced by T cells and NK cells. TNF- $\alpha$  urban production, observed by EF-2001 mice with no Sarcoma 180 solid tumor treatment, was higher than the TNF- $\alpha$  control levels when measured with the EF-2001-treated mice compared to (Fig. 6B). TNF- $\alpha$  has a different variety of operations on the target cell. It induces the expression of suppressing interleukin-mediated growth of tumor cells-2 receptor and the production of IFN- $\gamma$  from T cells. In addition, it also increases the antibody production by B cells. TNF- $\alpha$  has also been known to increase the cytotoxic activity of NK cells. Therefore, our results show that the production of TNF- $\alpha$  was increased in mice with cancer treated with EF-2001.

It was shown that tumor-bearing mice treated with EF-2001 expressed higher amounts of IgM than that of the control group (Fig. 6C). The enhanced level of IgM expression is possibly based on the enhancement of B cell capacity in the EF-2001 group, since premature B cells express an IgM as an antigen receptor, following activation of CD4 T cells. Finally, in mice bearing Sarcoma 180 tumors, the groups treated with EF-2001 showed higher levels of IgG production than the control groups (Fig. 6D). It is known that an increase in IgG level increased phagocytes and macrophages. IL-4 is known as an IgM/IgG shift-mediating interleukin. IFN- $\gamma$  and IL-4 collaboratively mediate Th1 and Th2 differentiations.

As the different tumor models (Ehrlich solid and Sarcoma180 carcinoma) have been assessed for anti-tumor activity of EF-2001, the tumor-inhibiting capacity of EF-2001 administration in Ehrlich solid carcinoma was approximately 83%, whereas it was approximately 60% in Sarcoma 180 carcinoma. This indicates that EF-2001 has a strong anti-tumor effect on the carcinoma and suggests that EF-2001 enhances the functions of macrophages and NK cells, thereby suppressing tumor proliferation. Also, the remarkable increase in TNF- $\alpha$  production suggests an activation of T cell activation and, thus suppressing tumor growth. Thus, the anti-tumor activity of EF-2001 might be based on the two different mechanisms of 1) direct anti-tumor activity and 2) indirect response to the host immune system. In the EF-2001 group, the expression levels of IFN- $\gamma$  and TNF- $\alpha$  via the cellular immune response were observed; however, the tumor-inhibiting effect was not strong. Therefore, it is considered that the TNF- $\alpha$  expression may elicit anti-tumor activity, as the possibility is supported by the production of TNF- $\alpha$  for the period [25]. Notably, *in vivo* TNF- $\alpha$  expression was reported to inhibit tumor growth and intravenous administration of TNF- $\alpha$  also inhibits tumor growth inoculated in mice intradermally [26]. For this issue, the TNF- $\alpha$  function in the anti-tumor capacity of EF-2001 can be further examined by several experiments using TNF- $\alpha$ -specific antibodies.

Because EF-2001 has been used an antifungal agent, it can be applied for leukemic patients among various cancers, who are at high risk of invasive fungal infections. EF-2001 as TNF- $\alpha$ -triggering agents can therefore applied with the dual therapeutic antifungal and anti-tumor activities. As previously reported [27], the enhanced anti-tumor chemotherapeutic potential in lung cancer patients was observed when amphotericin B was intravenously administered, suggesting the effect of EF-2001. However, clinical trials to develop



combination therapies involving EF-2001 require further immunological and pharmacological investigation in cancer patients.

## LIST OF ABBREVIATIONS

BRM	=	Biological response modifier
DPPH	=	1,1-diphenyl-2-picrylhydrazyl
ELISA	=	Enzyme-linked immunosorbent assay
IFN- $\gamma$	=	Interferon- $\gamma$
TNF- $\alpha$	=	Tumor necrosis factor- $\alpha$
NK	=	Natural killer

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

## HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

## CONSENT FOR PUBLICATION

Not applicable.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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