

Fermented Herbal Decoction Selectively Targeting Human Cancer Cell Line and Human Pathogenic Microorganism

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Abstract

Introduction: Prolonged immuno-suppressed status promised to induce internal growth of malignant cell and infectious agent, yet, only a small part of affected individuals seek medical attention or berried by commercially over-flowed fake information. Several studies have described complementary and alternative medicine as effective strategies for improving anti-infectious agent including malignant cell. The purpose of this study was to investigate the effect of a fermented herbal decoction (FHD) both *in vitro* and *in vivo* to malignant cells and microorganism by regulating leukocyte subset proportioning FHD as dietary material. **Methods:** In this approach of alternative study, selective anti-cancer effect by fermented decoction was tried to show first *in vitro* system both, cancer cell and virus strain. The fermented herbal decoction consisting of 80 sorts of herbs and fruits. The selective toxicity was set up and then for immunological factors in animal and human. The most important factor is to reduce side effect for a normal cell. **Results:** First, FHD was proved as safe by animal test. FHD regulated also the proportion of granulocyte and lymphocyte ratio both animal and human. *In vitro* culture showed selective toxicity by FHD against human melanoma and leukemia cell line but reduced toxicity was showed by normal cell line. As for the anti-virus activity, anti-virus effect was tested on the feeder layer of human fibroblast cell, after 9 days of culture. Second, FHD inhibits colon cancer growth in 3-methylholanthrene induced cancer in rat. **Conclusion:** The present results suggest that our fermented herbal decoction showed selective anti-cancer activities and anti-virus activities, together with the regulative effect on the immune system.

Keywords

Fermented Herbal Decoction, Human Malignant Cell Line, Human Normal Cell Line, Anti-Virus Activity, 3-Methylcholanthrene, Experimental Colon Cancer, *In Vitro*, *In Vivo* Anti-Cancer Trial

1. Introduction

Acquired immune deficient status refers to persistent or repeated episodes of clinically unexplainable fatigue that occur for a life related style. Prolonged fatigue is a common condition in the general population, while the prevalence of prolonged fatigue varies across communities and primary care settings, it is highly prevalent among working populations and young, adults and senile [1]-[11]. A Dutch Maastricht cohort study reported that 21.9% of working adults had prolonged fatigue. Another study indicated that 23.1% of patients visiting a primary medical institution showed prolonged fatigue, 50% of whom were between 30 - 49 years of age. Consistent with this finding, several studies have noted a high prevalence of chronic fatigue syndrome in adults [12] [13]. Fatigue not only affects daily life and social as well as occupational functions but is also detrimental to health in the long term, implicated as a cause of immune-deficient status, cancer and decreased QOL [9] [10]. Thus, prolonged fatigue is a condition that requires early prophylactic intervention and effective treatment by the instances reported [14]-[27]. So preventive medicine by so called CAM is required in this industrial world. There were many findings that reported in all over the world about anti-cancer agent. However, almost all the materials showed the cell toxicity against normal cell as well as cancer cell. In this report, the natural product had been known for anti-fatigue agent but recently reported the anti-cancer effect elsewhere [9] [10]. The trial had been made to clarify the FHD both *in vitro* and *in vivo* system. Numerous publications have focused on the mechanisms of pancreatic cancer development and progression associated with exposure to carcinogenic material, 3-methylecoranthrene famous carcinogen for colon cancer, and other tobacco-related carcinogens [5] [9] [10]. An immunocyte, especially NK cell has been identified as an important regulator of cell proliferation, apoptosis, migration, and angiogenesis in the most common epithelial human cancers [9], including cancer of the lung [28] [29], colon [30] [31], breast [32], oral cavity [33], stomach [34] [35], and pancreas [36]. It was initially thought that cellular responses after treatment of cancer cells with carcinogen were caused by intracellular signaling pathways activated immediately downstream of carcinogenesis [32]. However, emerging research suggests that most of these reported signaling events are in fact indirect responses caused by the carcinogen-mediated synthesis and release of neurotransmitters by cancer cells and the epithelia from which they arise [14]. In conventional western medicine, fatigue is treated with pharmacotherapy such as antidepressants or corti-

costeroids in order to mitigate or relieve symptoms that accompany fatigue; however, these medications are associated with various side effect. Additionally, despite the fact that a considerable proportion of the population exhibited prolonged fatigue [25]. Accordingly, many people who experience fatigue have turned to complementary and alternative medicine (CAM), and it was reported that a lot of people with fatigue symptoms use CAM, and the people with prolonged fatigue in the United States are attracted by CAM [26]. Among various CAM methods, fermented herbal decoctions; FHD have received substantial attention based on the fact that they are readily accessible and are generally perceived as safe. In fact, a previous clinical trial reported that fermented herbal decoctions significantly activated for complement system [37] [38] [39] [26] [27] [28]. So, this study to date has evaluated a fermented herbal decoction formulated in accordance with Traditional Alternative Medicine. The aim of this study was to conduct a clarification of the oral approach for regulate host immune system and against the parasite even in internal malignant cell itself.

2. Material and Methods

2.1. Animal Test for Material Safety Confirmation

Ten female eight-week-old ddY mice, were severed for the acute oral toxicity observation. The test systems were carried out according to Ethics of the Organization for Economic Co-operation and Development (OECD) Test Guideline 401. The mice were kept at $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$, 50% relative humidity in a SPF conditioned system.

2.2. Characterization of FHD *in Vivo*

Harmonization had been issued, (ICH)/WHO Good Clinical Practice standards (GCP) including certification by an external audit. The trial protocol has been approved by according to the Research Ethical Committee of Kanazawa Medical University. The assessments including a total number of leukocytes was ordered to count with blood chemical test for the medical diagnosis of public institution (Ishikawa Preventive Medicine Association, Ishikawa, Japan). In the differential counting, 200 cells were counted on a May-Grünwald-Gimsa stained slide, and percentages of lymphocytes and granulocytes were determined.

2.3. Study Design of Leukocyte and Leukocyte Subsets Assessment for Estimating FHD

We selected 30 healthy volunteers (mean age, 32.3 ± 7.8 years) and informed consented according to The Ethics Committee of Kanazawa Medical University. In addition, the experimental group was further divided into two groups according to the dosage variation. The peripheral blood obtained from the subjects by blood collection tube containing an anticoagulant EDTA-2K. (Becton Dickinson) as a secondary antibody and were incubated for 90 minutes at 4°C . After washing with PBS, +FCS, the grass adherent cells were added after washing with

PBS and served for the processing described in Section 2.5 After confirming safety test in mice [40] [41], human volunteers (n = 30) divided in to two groups that the grouping criteria by Abo *et al.*, lymphocyte rich group and granulocyte rich group [40] [41]. FHD were administered for one month and estimated blood cell before and after the trial. The constitution/condition dependent regulation in both in L rich type and G-rich type by administrating FHD, We reported that according to the lymphocyte subset content, lymphocyte rich type showed over 40% on the other hand granulocyte rich type show over 60% of granulocyte. Each type exhibited different character even in the same age, sexuality and each ager of individual. In the figure, within the same age and the sex, even in mankind can sorts out as G-rich type (granulocyte-rich) an/or L-rich type [29] [30] [31].

2.4. Regulation of Leukocyte and Leukocyte Subsets for Characterizing FHD as Immuno-Regulating Agent

After confirming safety test for mice [40], human volunteers (n = 30) divided in to two groups that the grouping criteria by Abo *et al.*, lymphocyte rich group and granulocyte rich group [41] [42] [43]. FHD were administered for one month and estimated blood cell before and after the trial. The constitution/condition dependent regulation in both in L rich type and G-rich type by administrating FHD, We reported that according to the lymphocyte subset content, lymphocyte rich type showed over 40% on the other hand granulocyte. After administrating FHD for one month, Both L-rich and G-rich types regulate for the intermediate value of lymphocyte and granulocyte ratio (**Table 1**).

2.5. Experimentally Induced Colon Cancer and FHD Application as CAM Therapy

Experimentally induced male rat colon cancer were induced by 3-methylcholanthrene; ECC (43). The group were divided into three four groups. They were Normal group, ECC, ECC+FHD. FHD were fur their divided according to their doses. Based on previous studies [44], the safety test were reported in the former reports [45].

2.6. Evaluation of FHD *in Vitro*

2.6.1. Reagents and Virus

TCDD was purchased from Radian International (Austin, TX 78720-1088, Catalog No. ED-901-B, Solution Lot: 34694-09). The chemical purity of this TCDD was 98% by chromatographic analysis. A goat anti-AhR polyclonal IgG antibody, N-19, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA 95060, USA). The Towne strain of CMV was used throughout the study, and has been described elsewhere [19]. CMV was propagated in MRC-5 cells, and the clarified supernatant stored in liquid nitrogen until use. Viral infectivity was titrated by plaque assay [32]. Cell culture. MRC-5 [32], and a human hepato-blastoma cell line, HepG2, purchased from the American Type Culture Collection, were cul-

tured in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Nissui Pharmaceutical, Tokyo) supplemented with 10% heat-inactivated fetal calf serum (FCS; Z. L. Bocknek Lab., Ont., Canada), L-glutamine (0.3 mg/ml), gentamicin (50 mg/ml), and amphotericin B (2.5 mg/ml). All cell cultures were maintained in a humidified incubator at 37 °C in 5% CO₂ /95% air. Viral production. When MRC-5 cells in 24-well plates (IWAKI Microplate, IWAKI Glass, Funahashi, Japan) reached confluency, the cells were inoculated with CMV at a multiplicity of infection (moi) of 0.1. After adsorption for 1 h, the cells were added with 1 ml of DMEM medium containing 2% fetal calf serum in the presence or absence of various concentrations of TCDD for the indicated time intervals. Production of infectious virus was titrated using a plaque assay [46] [47] [48] (Figure 1).

2.6.2. Human Cell Lines

An established human cell line were purchase from American Type Culture Collection (ATCC), A normal cell line and tumor cell line were prepared by ATCC and THP-1; Astrocytoma, U373MG; Human glioblastoma astrocytoma, MRC-5 ; Human embryo fibroblast (Normal Cell Line). First, FHD was diluted and prepared 10%, 1% and 0.1% as final concentration in RPMI-1640 plus FCS. Then each cell line were prepared 5×10^4 cell in each microplate well. The plate were incubated with 5% of CO₂ + 38c in CO₂ incubator. After 24 hrs incubation cultured cell were washed with 1 ml of PBS suspension. Trypan-blue dye (0.4%) was mixed to indicate the living cell in the suspension as dye exclusion test.

2.6.3. Regulation of Leukocyte and Leukocyte Subsets for Estimating FHD Character

After confirming safety test for mice, human volunteers (n = 30) divided in to two groups that the grouping criteria by Abo *et al.*, lymphocyte rich group and granulocyte rich group [14]. FHD were administered for one month and estimated blood cell before and after the trial. The constitution/condition dependent regulation in both in L rich type and G-rich type by administrating FHD, We reported that according to the lymphocyte subset content, lymphocyte rich type showed over 40% on the other hand granulocyte. Tis trial had been performed during May, 1st to May 31st, including peripheral blood collection.

After administrating FHD for one month, Both L-rich and G-rich types regulate for the intermediate value of lymphocyte and granulocyte ratio (Table 2).

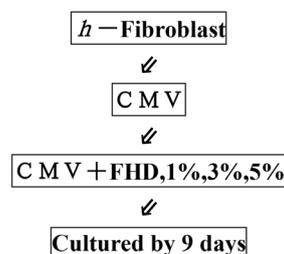


Figure 1. Study diagram *H*-Cytomegaro virus by FHD.

FHD showed cytotoxicity as 5%, 8% and 55% for human nerve cell line U373MG. However decreased cytotoxicity was shown to human normal cell line MEWO, showing tumor specific cytotoxicity. This FHD specific toxicity was confirmed another human tumor cell line, leukemia, melanome.

2.7. Statistical Analysis

Data were analyzed employing the 2002-2003 SAS Version 9.1 software (SAS Institute Inc. Cary, NC, USA) represented as means \pm standard deviations. The differences between MRY-treated and non-treated control were compared using a one-tailed analysis of variance. A P value < 0.05 was regarded to be statistically significant.

3. Results

3.1. Background Characteristics of FHD

3.1.1. *In Vivo* Experimental Immuno-Suppressed Mice and FHD Treatment

Forty individuals were assessed for eligibility and met the inclusion criteria. Twenty participants were randomized to each group. Five participants in the T group were lost to follow-up (two participants at four weeks and three participants at eight weeks); thus, 30 participants completed the study (**Figure 1**). The baseline characteristics of participants are summarized in **Table 1**. The mean age was 39.9 ± 2.6 years in the T group and 39.3 ± 6.0 years in the C group. The mean fatigue duration was 4.05 ± 1.23 months in the T group and 4.15 ± 1.18 months in the C group. The baseline characteristics were well balanced between the groups with regard to sex, age, body-mass index, drinking, smoking, and disease history ($P > 0.05$) (**Table 1**). The mean fatigue score at baseline was 20.0 ± 3.13 in the T group and 18.9 ± 4.22 in the C group, and secondary outcome measures at baseline including sleep quality, depression, and quality of life were not significantly different between the groups ($P > 0.05$) (**Table 1**).

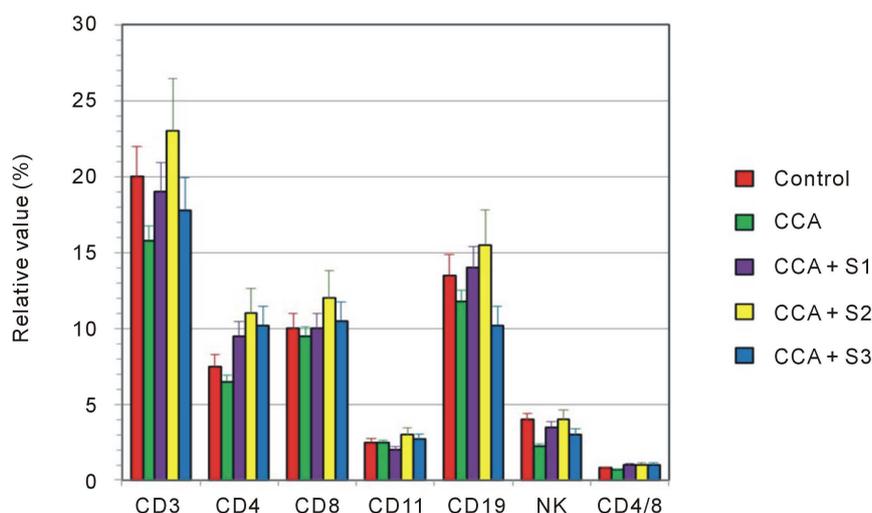
3.1.2. *In Vivo* Experimental Immuno-Suppressed Mice and FHD Treatment

In the animal model of immuno-competency reduction, mice were injected with MMC (5 mg/kg) to inhibit the bone marrow. An extract of FHD, which was breezed and dried, was administered orally at a dosage of 1g/kg/day for five consecutive days. In the combined treatment group with FHD + antibiotics, the number of bacteria decreased markedly. Moreover, in the joint treatment group, the blood bacteria number increase 6 hours and 12 hours after treatment and a great number of bacteria lasted after 24 hours. On the other hand, there was no increasing in bacteria count in the treatment group. After introducing MMC, additionally FHD was administered orally for five days. Two days after the last administration, spleen cells were removed, and phagocytic cells and lymphocytes were passively transferred into recipient mice whose bone marrows were inhibited (**Figure 2**).

Table 1. Constitution dependent regulation of lymphocyte by *FHD*.

CD	G type individual		L type individual	
	<i>FHD</i>		<i>FHD</i>	
	Before (%)	After (%)	Before (%)	After (%)
CD2	6.66	76.76	60.43	77.65
CD4	19.54	28.44	31.43	45.67
CD8	37.65	42.57	26.38	28.63
CD11	73.77	72.68	63.45	69.54
CD14	0.03	0.06	0.06	0.07
CD16	67.65	58.55	54.24	46.67
CD19	8.45	8.21	8.41	7.95
CD56	1.57	1.88	1.78	2.87

Constitution dependent regulation of leukocyte by *Rice Yeast*, Volunteer were divide according to their constitution base on their Lymphocyte Subpopulation expressed as CD positive Cell. The data represented the value obtained 30 days after MRY administration.



Fifty C57BL/6 mice were divided into five groups. Red group was control. Another four groups were administered cancer chemotherapeutic agent: CCA, Mitomycin-C, 5 mg/kg each mouse. S1 (violet) group was given 300 mg/kg of FHD, S2 (yellow) group was given 600 mg/kg of FHD and S3 (blue) group was 900 mg/kg. Ten days after the experiment, spleen cell were prepared and analyzed by CD positive lymphocyte.

Figure 2. *In vivo* experimental immuno-suppressed mice and FHD treatment.

3.1.3. *In Vivo* Experimental Anti-Tumor System in Immuno-Suppressed Mice and FHD Treatment

Sixty C57BL/6 mice were divided into two groups. One group was administered FHD 1g/kg for all the course of experiment. Another group was given same dose of FHD before fermentation. All the group were administered 3 methycoranthrene. Each week after the inducer administration, number of Colon cancer foci were macroscopically counted. As shown in **Table 2**, tumor foci in the intestine were different in the compared groups.

Table 2. The number of colon cancer foci in surface of colon and effect by FHD.

	FHD group (n = 30)	Control group (n = 30)	p-value
0 day	0	0	---
1 week	0	0	---
2 week	0	1.6 ± 0.1	0.473
3 week	0	3.5 ± 0.3	0.668
4 week	1.5 ± 0.3	12.3 ± 3.3	0.267

Sixty C57BL/6 mice were divided into two groups. One group was administered FHD 1g/kg for all the course of experiment. Another group was given same dose of raw material before fermentation. All the group were administered 3methycoranthrene. Each week after the inducer administration, number of Colon cancer foci were macroscopically counted.

3.2. *In Vitro* Experiment for Identifying FHD

3.2.1. Cytopathic Effect for Human Pathogenic Virus and Anti-Virus Activities by FHD

Direct evidence to human pathogenic vires, human cytomegaro virus was mixed with FHD in a different concentration of the decoction.

The culture system were observed through 9 days of the *in vitro* culture system, shown by photos in Figure three (**Figure 3(a)** to **Figure 3(f)**).

The result of anti CMV effect by FHD were Precisely reported in the photo regents under the results in photo.

3.2.2. Selective Cytotoxic Effect of Human Malignant Cell Line by FHD

So as to identify the Selective Cytotoxic Effect of Human Malignant Cell Line by FHD, FHD were directory mixed and cultured for 10 days. The human three different cultured cancer cell line were used in this experiment, Human Malignant Melanoma, Human Leukemia Cell Line and Human glioblastoma astrocytoma Cell Line and Normal Cell line.

First, FHD was diluted and prepared 10%, 1% and 0.1% as final concentration in RPMI-1640 plus FCS. Then each cell line were prepared 5×10^4 cell in each microplate well. The plate were incubated with CO₂ incubator. After 24 hrs incubation cultured cell were washed with 1 ml of PBS suspension. Trypan-blue dye (0.4%) was mixed to indicate the living cell in the suspension as dye exclusion test.

According to the culture test, FHD were showed specific cytotoxicity for either cancer cell line in at least three major cell line (**Figure 4(a)** to **Figure 4(c)**).

3.3. Safety Assessment

There was no specified events were found during the study period. In the FHD group, there were no significant mean changes in Biochemical parameter between baseline and post-treatment [49] [50].

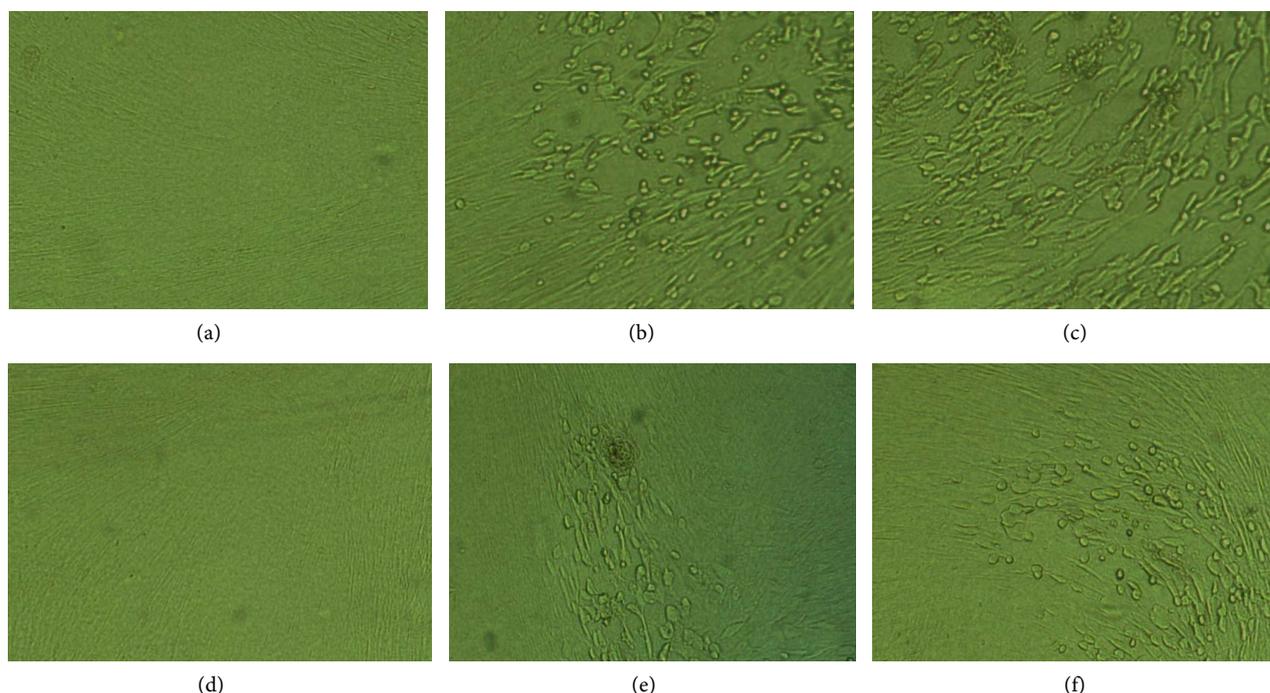


Photo Legend; (Microscopical Representation of anti-virus effect of HFD by x1 magnification by Digital Camera). (1) Photo of fibroblast layer before infected by HCMV; The layer was prepared for replication of HCMV; (2) Infected layer cell; The fibroblast layer on 9 days after infected by HCMV(PFU1000), indicating degradation of layer cells; (3) Infected layer cell; The fibroblast layer on 9 days after infected by HCMV(PFU2000), indicating severe degradation of layer cells; (4) Infected layer cell + 5%FHD; The fibroblast layer on 9 days after infected by HCMV(PFU1000)with 5%of FHD mixture, indicating no degradation of the layer, indicating no degradation of layer cells; (5) Infected layer cell+3%FHD; The fibroblast layer on 9 days after infected by HCMV(PFU1000)with 5%of FHD mixture, indicating severe degradation of layer cells; (6) Infected layer cell+1%FHD; The fibroblast layer on 9 days after infected by HCMV(PFU1000)with 5%of FHD mixture, indicating no degradation of the layer, indicating severe degradation of layer cells.

Figure 3. Cytopathic Effect by *h*-CMV and the Effect of FHD *in vitro*. (a) Huma Fibroblast Cell (HFC); (b) HFC+Human CMV (1000 moi); (c) HFC+Human CMV (2000 moi); (d) HCMV-infected + FHD (5%); (e) HCMV-infected + FHD (3%); (f) HCMV-infected + FHD (1%) + 1%.

4. Discussion

In this study, we compared and analyzed the effects of a fermented herbal decoction regimen on prolonged fatigue in young adults. Our results showed that the FHD had significant reductions in fatigue severity after the fermented herbal decoction intervention, and improved fatigue in the group were significantly different from the control group. Furthermore, improvements in fatigue were maintained simultaneous regulation of leukocyte subset proportion. Four weeks after completion of the intervention, suggesting that the fermented herbal decoction used in this study produced lasting beneficial effects in individuals with prolonged fatigue. In another study, four-week treatment of individuals with chronic fatigue with regulational effect for the blood cell and biochemical value listed for the evaluation of life related disease [11]-[17]. Fatigue is accompanied by various physical and psychological symptoms, including depression and sleep impairment. In the present study, fermented herbal decoction treatment also produced improvements in sleep quality; suggests that fermented herbal decoctions can also improve sleep problems that commonly accompany fatigue.

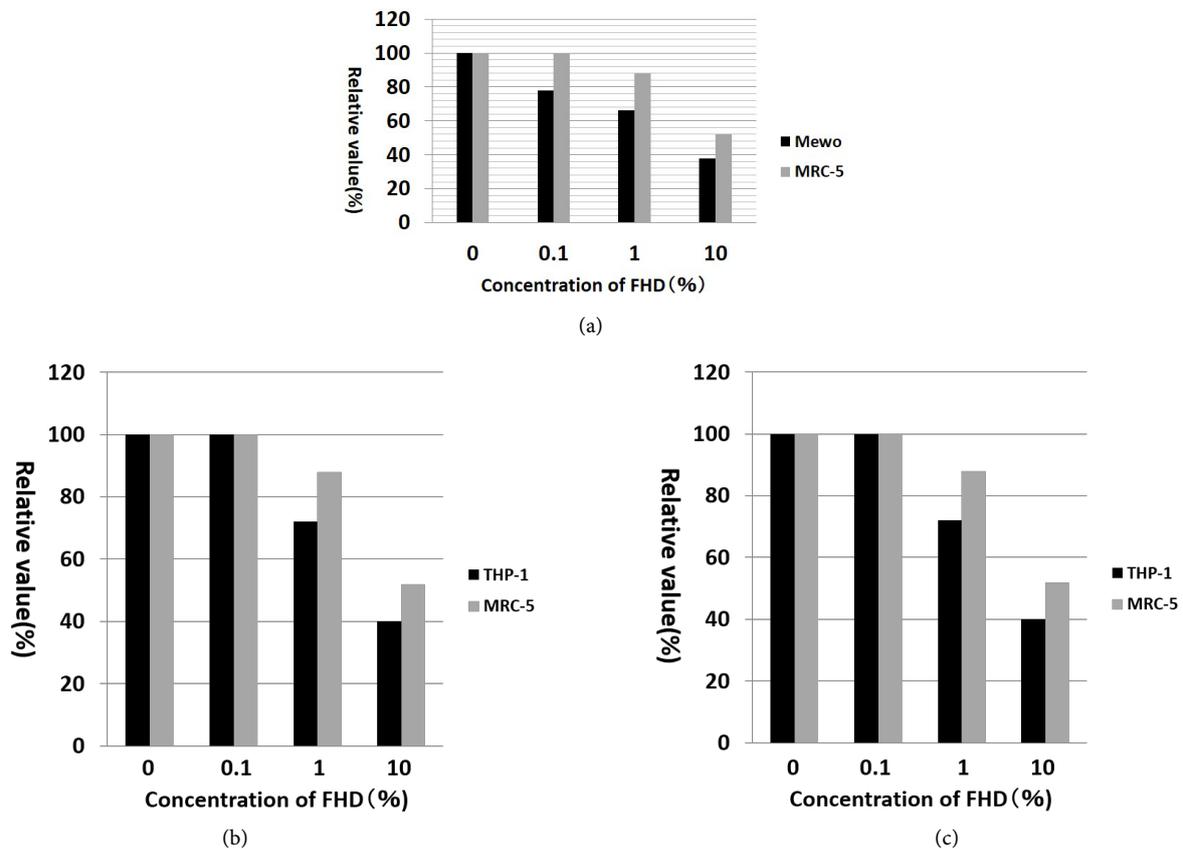


Figure 4. (a) Cytotoxic Effect for Human Malignant Melanoma Cell Line (Mewo) by FHD. An established human cell line were purchase from American Type Culture Collection (ATCC), A normal cell line and tumor cell line were prepared by ATCC. MRC-5; Human embryo fibroblast (Normal Cell Line). First, FHD was diluted and prepared 10%, 1% and 0.1% as final concentration in RPMI-1640 plus FCS. Then each cell line were prepared 5×10^4 cell in each microplate well. The plate were incubated with CO_2 incubator. After 24 hrs incubation cultured cell were washed with 1 ml of PBS suspension. Trypan-blue dye (0.4%) was mixed to indicate the living cell in the suspension as dye exclusion test; (b) Cytotoxic Effect for Human Leukemia Cell Line (THP-1) by FHD. An established human cell line were purchase from American Type Culture Collection (ATCC), A normal cell line and tumor cell line were prepared by ATCC, MRC-5; Human embryo fibroblast (Normal Cell Line). First, THP-1; Astrocytoma, was diluted and prepared 10%, 1% and 0.1% as final concentration in RPMI-1640 plus FCS. Then each cell line were prepared 5×10^4 cell in each microplate well. The plate were incubated with CO_2 incubator. After 24 hrs incubation cultured cell were washed with 1 ml of PBS suspension. Trypan-blue dye (0.4%) was mixed to indicate the living cell in the suspension as dye exclusion test; (c) Cytotoxic Effect for Human glioblastoma astrocytoma Cell Line (U373MG) by FHD. An established human cell line were purchase from American Type Culture Collection (ATCC), A normal cell line and tumor cell line were prepared by ATCC and U373MG; Human glioblastoma astrocytoma, MRC-5; Human embryo fibroblast (Normal Cell Line). First, FHD was diluted and prepared 10%, 1% and 0.1% as final concentration in RPMI-1640 plus FCS. Then each cell line were prepared 5×10^4 cell in each microplate well. The plate were incubated with CO_2 incubator. After 24 hrs incubation cultured cell were washed with 1 ml of PBS suspension. Trypan-blue dye (0.4%) was mixed to indicate the living cell in the suspension as dye exclusion test

Additionally, improvements in sleep quality were still evident at four weeks after treatment completion, similar to the pattern observed for improvements in fatigue. Further, the ultimate objective of this study was to evaluate the other character of this decoction for cancer cell and pathogenic virus. Several reports describe CAMs as effective strategies for reducing fatigue. A systematic review of randomized controlled trials of various CAMs in patients with cancer and immunosuppressive virus diseases. However, this FHD could not showed the an-

ti-bacterial activity, showing this FHD had a different mechanism of anti-bacterial chemotherapeutic agent such as antibiotics [14]. As for the cytotoxicity of FHD to malignant cancer cell line and pathogenic virus were eminent effect for especially for human tumor cell line. There are many findings were found in all over the world for anti-cancer agent, but almost all the material exhibited a toxicity both for cancer cell as well as normal cell. The natural product had been known for anti-fatigue agent but recently reported the anti-cancer effect elsewhere [51]-[78]. Finally, our fermented herbal decoction was obtained from 80 plant sources, such that there were numerous active components potentially mediating the observed efficacy. It will be useful in future studies to identify these active compounds and obtain pharmacokinetic data.

5. Conclusion

This study found that FHD for 4 weeks regulated a lymphocyte and granulocyte proportion. The fermented herbal decoction intervention also significantly improved anti-cancer cell line yet for normal cell line *in vitro*, which is a common wild herb ragweed. The more complete effect was showed for human cytomegalovirus.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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