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Anti-cancer activity comparisons of aqueous extracts from *Inonotus obliquus*, *Cordyceps militaris* and *Uncaria tomentosa in vitro* and *in vivo*

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ABSTRACT

The previous study was to investigate the anti-cancer activity of aqueous extracts from *Inonotus obliquus* (IO), *Cordyceps militaris* (CM) and *Uncaria tomentosa* (UT). The antioxidant and immunomodulatory activities of aqueous extracts were measured and compared. The results showed UT exhibited excellent SOD-like activity (113.1 U/mg) and stronger DPPH radical scavenging activity (EC_{50}, 0.442 mg/ml). Moreover, inhibition ratio of U2OS cells was reached to 92.17% at 7 mg/ml for 72 h. In addition, BALB/c mice were inoculated with DLD-1 cells and fed with three samples to be determined weight, tumor diameter and tumor necrosis factor (TNF) α. UT also performed marvelous anti-cancer activity on inhibition ratio of weight (44.2%) and tumor diameter (48.12%) and TNF-α concentration (69.23%). All these results indicated that aqueous extract from UT exhibited potent anti-cancer activity and also could provide a theoretical basis for utilization as a nutritious function material.

Keywords: Uncaria tomentosa, antioxidant activity, immunomodulatory activity, anti-cancer activity, TNF-α

1. Introduction

Inonotus obliquus (IO), also known as chaga mushroom [1], is a white-rot medicinal fungus belonging to the family Hymenochaetaceae of Basidiomycetes [2], which habits as a parasitism on birches in the cold latitudes of Europe and Asia [3], where IO had been used as a traditional remedy to cure various diseases such as cancer, cerebrovascular diseases, diabetes, gastrointestinal diseases since the sixteenth century [4]. In recent years, many polyphenolic compounds, triterpenoids, polysaccharides and steroids, such as lanosterol, inotodiol, trametenolic acids, and ergosterol peroxides had been isolated from Inonotus sclerotia, which shown various biological activities, including hypoglycemic [5], anti-inflammatory ability [6], antioxidant effect [7], hepato-protective [8], and anti-tumor [9], anti-inflammatory ability effects [10].

Cordyceps militaris (CM), a caterpillar-shaped Chinese traditional mushroom [11] belonging to the class ascomycetes, has a similar physiological effect to Cordyceps sinensis [12]. CM contains abundant kinds of active components such as cordycepin, adenine, cordycepic acid, adenosine, cordyheptapeptide, polysaccharide [13, 14]. Owing to its various physiological activities, CM has been extensively used in East Asia for treatment of anti-inflammation [15], renal dysfunction [16], asthma [17], and cancer [18].

Uncaria tomentosa (UT), popularly known in English as Cat's Claw [19], is a lignified vine that grows in the tropical areas South and Central America liana classified into Rubiaceae family [20], which has been used by Peru and other South American countries to treat many diseases including asthma, arthritis and other inflammatory diseases [21, 22, 23] due to its proven immunostimulatory and anti-inflammatory activities as well as its anticancer and antioxidative effects validated by numerous experimental studies in recent years [24].

Tumor necrosis factor-alpha (TNF-α), first identified as an antitumor agent [25],

now also is acknowledged as a tumor-promoting cytokine that links inflammation and cancer in the central nervous system (CNS) [26]. It is produced primarily by macrophages in response to bacteria, viruses other external stimulation [27]. TNF-α is cytotoxic to lymphocytes and in some situations can take measures to suppress the immune system [28].

Although anti-inflammatory and antioxidant activity and other bioactivities of three materials have been studied individually, limited studies were focused on the comparison of anti-cancer activity of a variety of materials. In this study, the purpose of the present study was to establish a complete detection system for aqueous extracts from *Inonotus obliquus*, *Cordyceps militaris* and *Uncaria tomentosa* for their anti-cancer properties. Three kinds of aqueous extracts were comparatively investigated in antioxidant activity, anti-proliferation activity and anti-tumor activity.

2. Material and methods

2.1 Chemicals and reagents

Ascorbic acid was purchased from Wako Pure Chemical Osaka, Japan. SOD Assay Kit-WST according to the technical manual provided by Dojindo Molecular Technologies, Inc. 2,2-diphenyl-1-picry-hydrazyl (DPPH), minimal essential medium eagle medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin solution were purchased from Sigma Aldrich, Inc. (Saint Louis, MO, USA). MTT stock solution (5 mg/ml in D-PBS filtrated by 0.2 um filter) and dimethyl sulfoxide (DMSO) were supplied by National Institute of advanced industrial science and technology, AIST, Japan. TNF-α EIA Kit (IM1121) was purchased from IMMUNOTECH, Japan. All other chemicals and solvents were analytical grade and utilized without further purification.

2.2 Cell lines

Human normal fibroblast cells line (TIG-3) and Human osteosarcoma cell lines (U2OS) were obtained from Japanese Collection of Research Bioresources (JCRB) Cell Bank and maintained in DMEM containing 10% (v/v) FBS and antibiotics (consisting of 100 U/ml penicillin and 100 µg/ml streptomycin) at 37 °C in 5% (v/v) CO_2 atmosphere. Cells were cultured for 2-3 days to reach the logarithmic phase and utilized for experiments.

2.3 Animals and experimental design

Four-week-old female BALB/c nude mice weighing 18.4±2.0 g and DLD-1 human colon cancer cell line was obtained from the Cell Resource Center for Biomedical Research, Aging and Cancer, Tohoku University (Japan). All mice were randomly divided into 4 groups consisting of five per cage and fed standard laboratory chow with 12-h dark/light cycle conditions for 1 week before the start of the experiments with a constant temperature of 20±2 °C and humidity, 60±5%. All laboratory feed pellets and bedding was autoclaved and supported by Laboratory Animal Resource Center, University of Tsukuba (Japan).

2.4 Plant Material and Preparation of Extract

CM (mycelium), IO, UT were collected by Asia Environmental Laboratory Co., Ltd. Oita, Japan. Raw materials were washed thoroughly with distilled water and dried in a convention oven at 50 °C for 72 h. All of them were smashed by an impeller type pulverizer (1029-B type, Yoshida Seisakusho Co., Ltd., Japan). The powder (2.0 g) was extracted by distilled water (1 L, 3 times and 2 h). Each extract was subsequently filtered through a Whatman GF/A filter paper, and centrifuged at 7500 rpm for 30 min at ambient temperature. The supernatant was concentrated in a rotary evaporator at 60 °C and removed free protein layer by the use of method of Sevage. Ultimately concentrated solutions were lyophilized to get powder (w/w) after treated with vacuum freeze drying under reduced pressure. The aqueous extract powders were stored at -20 °C until further utilization.

2.5 Assay for antioxidant activity

2.5.1 SOD-like activity

SOD-like activity of the extracts was measured by the SOD Assay Kit-WST according to the technical manual provided by Dojindo Molecular Technologies, Inc. Firstly, 20 µl of sample solution was added to blank 2 well and each sample, meanwhile 20 µl of double distilled water was added to each blank 1 and blank 3 well in a 96-well plate. After that, 200 µl of WST working solution was added to each well, mixed waiting for further processing. Afterwards, 20 µl of dilution buffer was added to each blank 2 and blank 3 well, and 20 µl of enzyme working solution was added to each sample and blank 1 well. The plate was incubated at 37 °C for 20 min and the optical density (OD) was measured at 450 nm using a micro-plate reader (Bio-Rad Model 550, USA). The SOD-like activity was calculated by the following equation:

$$\text{SOD activity (inhibition rate \%)} = \{[(A_{\text{blank1}} - A_{\text{blank3}}) - (A_{\text{sample}} - A_{\text{blank2}})] / (A_{\text{blank1}} - A_{\text{blank3}})\} \times 100\%$$

Where, A_{blank1}, A_{blank2}, A_{blank3} and A_{sample} were the absorbance of $blank_1$, $blank_2$, $blank_3$ and sample wells. 1 unit of SOD activity was defined as the amount of enzyme having a 50% inhibitory effect on WST-1.

IC_{50} value (mg/ml) was the effective concentration at which SOD activity was scavenged by 50% and was calculated by interpolation from a linear regression analysis.

2.5.2 DPPH radical-scavenging activity assay

DPPH radical-scavenging activities of aqueous samples were measured in accordance with the method described by Blois with some modifications [31]. Aliquots (0.5 mL) of diverse concentrations of raw materials were mixed with 2 mL (25 µg/mL) of a MeOH solution of DPPH. The mixture was shaken vigorously and allowed to stand in the dark for 30 min. The absorbance was measured with a spectrophotometer (Lambda35, Prekin Elmer Co. Ltd., USA) at 517 nm against a blank. Decrease of the DPPH solution absorbance indicated an increase of the DPPH radical-scavenging activity. Ascorbic acid was used as positive controls. DPPH free radical-scavenging activity was calculated according to the following equation:

$$\text{DPPH radical-scavenging activity (\%)} = [A_0 - A_1/A_0] \times 100\%$$

Where A_0 is the absorbance without samples and A_1 the absorbance in the presence of the samples.

IC_{50} value (mg/ml) was the effectual concentration at which DPPH radicals were scavenged by 50% and was obtained by interpolation from a linear regression analysis.

2.6 Cell evaluation

2.6.1 Non-toxicity effect on human normal fibroblast cells TIG-3

Non-toxicity effect of different aqueous extracts was estimated using the MTT assay. Briefly, TIG-3 cells were cultured in DMEM at 37 °C in a 5% CO_2 atmosphere to logarithmic phase. The cells were harvested and an aliquot (100 µl) of TIG-3 cells suspension (2×10^4 cells/ml) was dispensed into a 96-well plate (2×10^3 cells/well) and pre-incubated at 37 °C in a 5% CO_2 atmosphere for

24 h. Then cells were exposed to aqueous extract (7 mg/ml) for 72 h and in order to identify the optimum concentration and eliminate the toxicity of the sample itself, another sample (0.7 mg/ml) was also treated for 72 h. After drugs exposure, 96-well plate was removed from the incubator and 20μl MTT stock solution was added to each well incubated at 37 °C, 5% CO_2 for 4 h. Afterwards, 96-well plate was removed from the incubator and aspirated the solution and further added 100 μl DMSO to each well and rotated the plate for 5 min to distribute evenly. Ultimately, absorbance was measured using an ELISA reader at 540 nm.

2.6.2 Anti-proliferation effect on Human osteosarcoma cells U2OS

Anti-proliferation effect of different aqueous extracts was determined by the MTT assay and specific experimental procedures were basically consistent except cells changed from TIG-3 to U2OS.

2.7 Immunomodulatory evaluation

2.7.1 Weight of mice and tumor diameter.

At first week, mice were randomly divided into 4 groups with 5 mice per group and the nude mice were fed with three distinct samples respectively (positive control) and distilled water (negative control) 0.2 ml per day. At day 8, the nude mice were injected subcutaneously into the right groin with 0.2 ml DLD-1 cancer cells (5×10^6 cells/ml). Afterwards, each group was intragastric administrated continuously for 4 weeks meanwhile the changes of mice body weight and tumor size were measured. The inhibition ratio of weight was calculated by the following formula:

$$\text{Inhibition ratio (\%)} = \{1- (W-W_T)/(W-W_C)\} \times 100\%$$

Where W is the weight of untreated mice and W_C is the weight of control and W_T is the weight of treated groups. This study was in quintuplicate.

The inhibition ratio of tumor growth was calculated by the following formula:

$$\text{Inhibition ratio (\%)} = (TD_C - TD_T)/TD_C \times 100\%$$

Where TD_C is the tumor diameter of control and TD_T is the tumor diameter of treated groups. This study was in quintuplicate.

2.7.2 Microcosmic mice experiment: blood analysis for rat anti-cancer inhibitory activity.

TNF-α EIA Kit (IM1121) method was directed by IMMUNOTECH guidance. Firstly, add 100 μL of conjugate per well and 100 μL of calibrator, or sample and incubate for 2 h, at ambient temperature while shaking. After that, the wells were washed and added 200 μL of substrate, incubated 45 min at ambient temperature in the dark, while shaking. Add 50 μL of stop solution. Read absorbance at 405 nm concentrations in mice with OD450 inverse proportion between the values, by reference to article drawing standard curve.

2.8 Statistical analysis

All values are means of at least three replicates ± S.D. Differences in mean values between groups were analyzed by a one-way analysis of variance (ANOVA) and Student's t-test using SPSS statistical software (version 16.0 for Windows, SPSS Inc., Chicago, IL, USA) to determine significant differences among means ($P<0.05$).

3. Result and discussion

3.1 Assay for SOD-like activity

Table 1: SOD activity and DPPH scavenging radical activity determined of three biomaterials.

Sample	SOD IC_{50} (mg/ml)	SOD vigor U/mg	DPPH IC_{50} (mg/ml)
Cordyceps militaris	1.958	25.536	4.624
Inonotus obliquus	0.462	108.225	1.838
Uncaria tomentosa	0.442	113.122	0.612

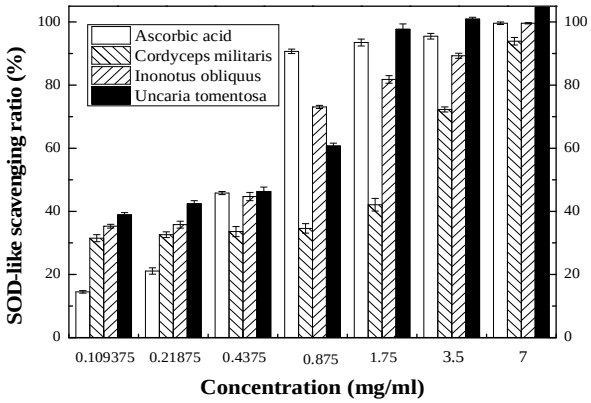

Fig 1: SOD-like activity of aqueous extracts of three biomaterials at various concentrations

Concentration was followed by geometric gradient and the results were in Fig. 1. SOD-like activity of all the three samples constantly increased which could be concluded that three kinds of samples were totally in a dose-dependent manner. SOD-like scavenging ratios of CM, IO and UT at the concentration of 7 mg/ml were 93.9%, 96.6% and 100%, respectively. From Table 1, IC_{50} of CM, IO and UT were 1.958 mg/ml, 0.462 mg/ml and 0.442 mg/ml, respectively. Also SOD vigor of CM (25.536 U/mg), IO (108.225 U/mg) and UT (113.122 U/mg) were calculated. On concrete analysis, at a relatively minimal concentration range, from 0.109375 to 0.4375 mg/ml, three samples were marginally higher than ascorbic acid. Nevertheless, ascorbic acid was higher than samples except UT at higher densities. Hence, it was demonstrated that UT possessed significant SOD-like activity compared with other two materials, whether on activity or effectiveness. Among the substrates, the performance of UT was the best, while that of CM was the worst.

3.2 Assay for DPPH radical scavenging activity

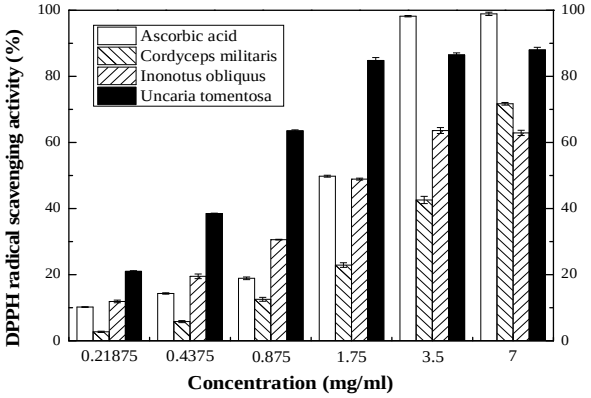

Fig 2: DPPH radical scavenging activities of aqueous extracts of three biomaterials at various concentrations

DPPH radical scavenging activities of three biomaterials were illustrated in Fig. 2, and from that, the scavenging ratio of aqueous extracts at 7 mg/ml on DPPH radicals were 71.7%, 62.9% and 88.0% for CM, IO and UT, respectively. Meanwhile, IC_{50} of CM, IO and UT were 4.624 mg/ml, 1.838 mg/ml and 0.612 mg/ml, respectively from Table 2. Three materials inhibited significant radical activity and were in a dose-dependent manner. Nevertheless, the free radical scavenging ability of aqueous extraction from CM was the most inconspicuous. Specifically, DPPH radical scavenging activity of UT increased drastically when the concentrations of aqueous extracts were between 0.22 and 1.75 mg/ml in a dose-dependent manner, and reached a plateau after 2.5 mg/ml, among these indexes. Meanwhile, other two materials were raised relatively slowly until 7 mg/ml. This result was coherent with previous SOD-like scavenging activity consequence, which further testified aqueous extract of UT exhibited higher antioxidant activity. Preceding studies indicated that ascorbic acid and polyphenols exhibited potent DPPH radical scavenging activity, those conjugated with polyphenols such as ferulic acid, have been shown to possess such activity, which implicated aqueous extracts of three materials involving abundant polyphenols and other antioxidant ingredients.

3.3 Non-toxicity effect on human normal fibroblast cells TIG-3

The consequences of non-toxicity on TIG-3 human normal fibroblast cells were shown in Fig. 3. In this study, various concentrations (0, 0.7, 7 mg/ml) were observed. It easily concluded that absorbance of samples was not decreased dramatically and on the contrary the cells growth curves of three samples were almost consistent with the curve of TIG-3 normal cells, which meant cells viabilities exhibited no significant change in response to the addition of various concentrations of aqueous extract treated. In other words, aqueous extracts from IO, CM and UT, belonging to natural materials, were non-toxic which made it possible for absorption and utilization by human beings.

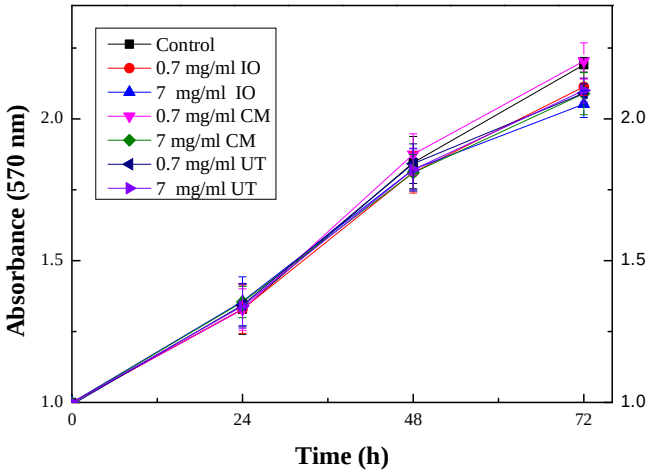

Fig 3: Non-toxicity effect on human normal fibroblast cells TIG-3 for 72 h

3.4 Anti-proliferation effect on human osteosarcoma cells U2OS

The anti-proliferation effect of aqueous extracts from CM, IO and UT on the growth of U2OS cells was investigated and the results were shown in Fig. 4. Besides, 0.7 mg/ml of three extracts were exposed to U2OS cells as well as to avoid interference from the drugs. Three aqueous extracts showed anti-proliferation activity in a dose-dependent manner and extract from UT was exhibited stronger than other two kinds of materials, which inhibition ratio was 68.19% at the concentration of 0.7 mg/ml. Furthermore, at the concentration of 7 mg/ml, the inhibition ratio was achieved to 92.17%. For CM and IO, inhibition ratio was 69.96% and 88.98% at the concentration of 7 mg/ml. The results were corresponded with preceding study of assay for antioxidant, which further proved high bioactivity.

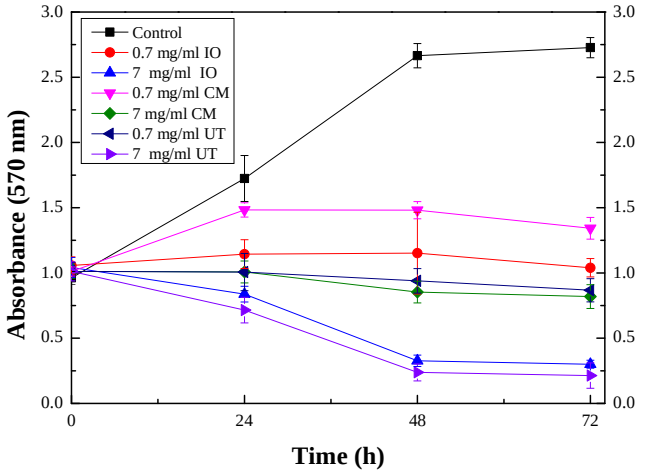

Fig 4: Anti-proliferation effect on the growth of human osteosarcoma cells U2OS for 72 h.

3.5 Weight of mice and tumor diameter

The results of anti-tumor activities of aqueous extracts from three samples against DLD-1 tumor-bearing nude mice *in vivo* are summarized in Table 2. Weight and tumor size and these two indexes trends were displayed in Fig. 5 and Fig. 6. Anti-tumor activities were considerably observed in three samples on weight and tumor diameter. At the beginning after injection, there was no perceptible change on both weight and tumor size. However, during day 24 to day 28, mice weight decreased smoothly and tumor size rose steadily contrast with the control group. On day 36, compared with negative control group, the weight of mice was decreased smoothly due to be fed with aqueous extracts, CM, IO and UT were 24.8, 25.4 and 26.3 g, compared with 24.4 g of negative control and inhibition ratio were 9.3%, 22.7% and 44.2%, respectively. The normal physiological metabolism and growth of mice at a certain time should be taken into account, which meant inhibition ratio of three materials on actual weight must be higher. Meanwhile, the average tumor diameter of negative control mice was 13.3 mm and the tumor diameter of mice fed with three samples (totally 7 mg/ml) were 10.1, 8.9 and 6.9 mm for CM, IO and UT, respectively. In other words, the tumor diameter growth inhibition ratio was 24.06, 33.08 and 48.48%, respectively. Hence, these three samples were truly potential bio-material resources to postpone cancer attack and complication occurrence. For instance, during day 24 to day 28, a conspicuous change has appeared not only in weight but also in tumor size. It was worth noting that cancer-induced weight loss is caused by changes in the metabolism of protein, carbohydrates, and fat, causing the body to burn calories faster than they can ordinarily be replaced, so as to simply eating or drinking more is not enough to slow down or reverse weight and muscle loss. Thus, the consequences above were in accordance with this conclusion as well. Among these three samples, aqueous extract from UT showed equivalently high activity, which also was consistent with the effect of antioxidant experiment.

Table 2: Effect of three materials on the body weight and tumor diameter of BALB/c nude mice

Sample	Weight(Before, After 4 weeks) (g)		Inhibition ratio %	Tumor diameter (mm)	Inhibition ratio %
Control	28.8 ± 0.7	24.4 ± 0.6		13.3 ± 0.2	
Cordyceps militaris	28.7 ± 0.6	24.8 ± 0.8	9.3	10.1 ± 0.1	24.06
Inonotus obliquus	28.8 ± 0.6	25.4 ± 0.8	22.7	8.9 ± 0.2	33.08
Uncaria tomentosa	28.7 ± 0.5	26.3 ± 0.9	44.2	6.9 ± 0.3	48.12

N=5; weight ± S.D

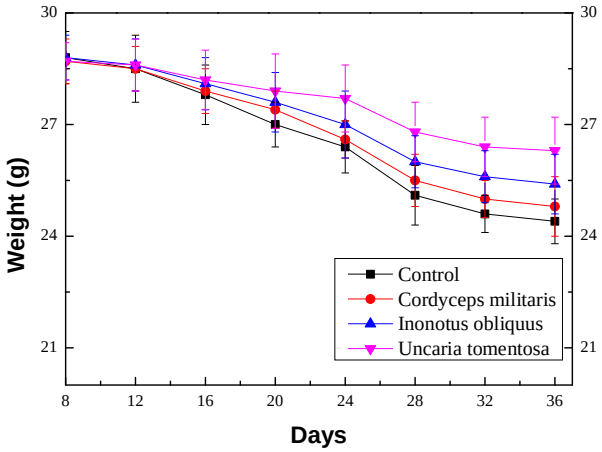

Fig 5: Weight changes of rude mice treated with aqueous extracts of three biomaterials

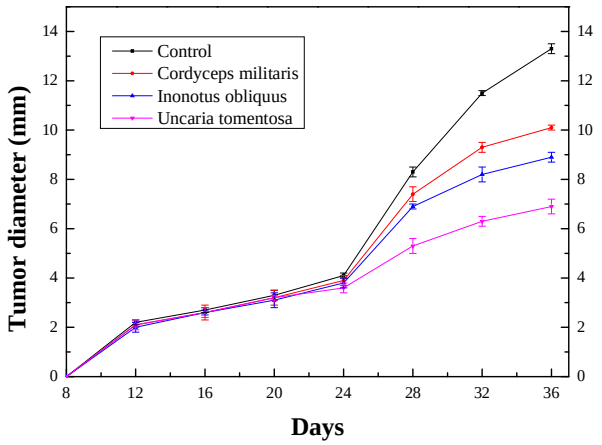

Fig 6: Tumor diameter changes of rude mice treated with aqueous extracts of three biomaterials.

3.6 Assay for TNF-α concentration of mice

The stimulative effect of three aqueous extracts on TNF-α concentration was illustrated in Fig. 7. The results showed that, TNF-α concentrations of control group, CM, IO and UT were 150.8, 123.2, 72.5, 46.4 pg/ml. Previous studies have demonstrated that CM, IO and UT have the capacity to inhibit the secretion of the pro-inflammatory cytokine, TNF-α [30, 31, 32] which was coherent with the experimental consequence. Moreover, inhibition activity

of three kinds of aqueous extracts was compared and among these three materials, UT (46.4 pg/ml, inhibition ratio 69.23%), was the most observable effect on the reduce of TNF-α concentration.

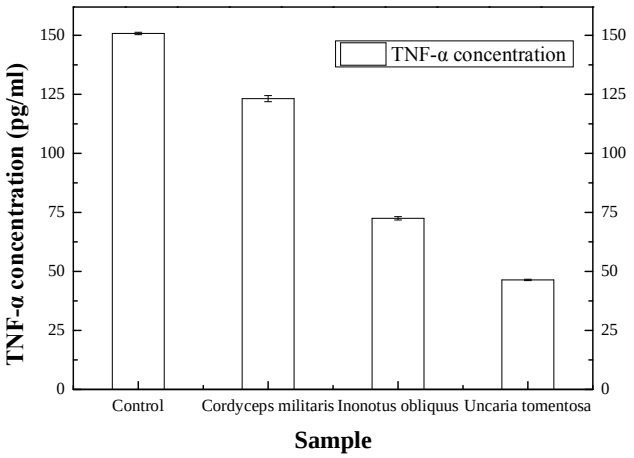

Fig 7: TNF-α concentration of mice

4. Conclusion

The present study reveals that antioxidant activity and anti-cancer activity of aqueous extracts from *Inonotus obliquus*, *Cordyceps militaris* (mycelium) and *Uncaria tomentosa* were investigated in vitro and *in vivo*. Three biomaterials all showed admirable effect on SOD-like activity and DPPH radical scavenging activity, non-toxicity on human normal fibroblast cells, anti-proliferation on human osteosarcoma cancer cells. In addition, weight decrease and tumor growth were inhibited as well. Among three biomaterials, UT was the optimal resource for anti-inflammatory and anti-cancer effects in mice, which might be utilized for the treatment of cancer. Further works are in progress on the isolation, purification, characterization of aqueous extract from UT and investigated the mechanism of anti-cancer activity.

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