The effects of solutions of maca (*Lepidium meyenii*) powder as a food/feed supplement on the viability of murine macrophage cells by digital image analysis

ABSTRACT

Maca (*Lepidium meyenii*) is a tuber root plant and belong to Brassicaceae family and recently used as a supplement in human and animal nutrition. In this study, it was aimed to investigate the cytotoxicity of two solutions (aqueous and ethanol) of maca root powder by digital image analysis. Maca powder was mixed in ultra distilled water and ethanol (1:2 w/v) for 24 h at 4 °C. The mixtures were centrifuged and the supernatants were ten-fold diluted for cytotoxic analysis of Raw 264.7 murine macrophage cells. After seeded the cell cultures in microplates, ten-fold dilutions (from 10^{-1} to 10^{-7}) of both maca solutions were added as six replicates for 24 h. While the aqueous maca solution increased the number of dead cells at 10^{-1} (50 mg mL^{-1}), the ethanolic solution statistically increased the number of dead cells at 10^{-1}, 10^{-2} and 10^{-3} (50, 5 and 0.5 mg mL^{-1}) ($P<0.01$). In conclusion, the alcoholic preparation of maca powder caused a higher cytotoxic effect on the murine macrophage cells than the aqueous preparation due to the solvents and the dilution factor in this study.

Keywords: *Lepidium meyenii*, Food/Feed Supplement, Cytotoxicity

INTRODUCTION

Maca (*Lepidium meyenii*) is a tuber root plant and belong to Brassicaceae family such as broccoli, cabbage and turnip. On both human and animal nutrition, many researches were published about the effects of maca (Korkmaz et al., 2016; Bilal et al., 2016; Gonzales et al., 2020), its extracts and bioactive compounds such as polysaccharides, macamide, macaene, phenols and glucosinolates (Korkmaz, 2018; Gonzales et al., 2020). So, there are many products in various forms of maca as food and feed supplements for the consumptions of human and animals. Therefore, it was reported that the maca extracts had antioxidant, free-radical scavenging, cell viability and cytotoxic effects in vitro studies. These effects vary due to the extraction methods and the most used in the studies are aqueous and alcoholic extractions methods (Rodríguez-Huamán et al., 2017; Ye et al., 2018).

Besides to the methods of biochemical, colorimetric and spectrophotometric analysis, the methods of digital image analysis were recently developed to quantify the cell viability and the cytotoxicity for both *in vivo* and *in vitro* studies (Collins, 2007; Sandhya, 2011). The advantages of these methods were to be rapid, economic and pollution-free (Grishagin, 2015; Freitag et al., 2020).

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Research Article

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In this study, the aim was to investigate the effect of aqueous and alcoholic solutions of maca powder on the cell viability using image analysis.

MATERIAL and METHOD

Materials

Ultra-pure water was from PURELAB flex 3, Elga, United Kingdom. Ethanol (%99.8, CAS 64-17-5) and trypan blue solution (0.4%, sterile-filtered) was purchased from Sigma-Aldrich, Germany. DMEM (500mL, Dulbecco's Modified Eagle Medium (w/o L-glutamine, w/o sodium pyruvate), L-alanyl-L-glutamine (200 mM), penicillin (10,000 units mL^{-1})-streptomycin (10 mg mL^{-1})-amphotericin B (0.025 mg mL^{-1}) solution, foetal bovine serum (FBS, European grade), trypsin-EDTA solution (w/o phenol red) and Dulbecco's Phosphate Buffered Saline (PBS w/o calcium magnesium) were obtained from Biological Industries, USA. For cell culture, the 75 cm^2-flasks were from EasYFlask, Thermo Scientific and 96-well microplates were obtained from CellStar, Greiner Bio-One, Germany. The syringe filters (sterile, 0.22 µm) was purchased from Merck Millipore, Germany. Sterile centrifuge tubes were from ISOLAB, Germany.

Plant material and preparation

Organic maca (*Lepidium meyenii* Walp.) root powder was cultivated in Junin and imported from Peru. Maca powder samples were added to ultra-pure water (1:2, w/v) (MW) or ethanol (1:2, w/v) (ME) and mixed well. Then, they were kept at 4 °C overnight. After centrifuged at 3500 rpm for 15 min, both supernatants were filtered using the sterile syringe filters (0.22 µm) and stored in the sterile tubes at 4 °C for further analysis.

Cell culture

Raw 264.7 murine macrophage cells were from American Type Culture Collection (ATCC), Manassas, VA, USA). Serum heat-inactivation was performed in a water bath at 56 ◦C for 30 minutes before use. All solutions were heated to 37 °C before the process of cell cultivation. The cells were cultured in DMEM supplemented with 10% inactivated foetal bovine serum, L-alanyl-L-glutamine and 1% penicillin-streptomycin at 37 °C. It was maintained in cell culture flasks (75 cm^2) in the incubator with the condition of 37 °C and 5% CO_2 for 24 h to confluence. After the incubation, all waste medium was discarded and the cell monolayer was disaggregated with trypsin-EDTA in the incubator for 3 min. The suspension was centrifuged with a refrigerated centrifuge at 1200 rpm/15 min. the supernatant was discarded and the pellet was suspended in fresh medium. Then, viable and dead cells were counted by the method of trypan blue (0.4%) staining with a haemocytometer as in the section of Cell Staining and Image Analysis. For the preparation of 96-well microplates, 100 µL of the stock viable cell suspension (3 x 10^5 cell mL^{-1}) was seeded in each well (3 x 10^4 cell) and kept in the incubator for 24 h to confluence at least of 90%.

Cytotoxicity assay

Both of maca solutions (MW and ME) was 10-fold serially diluted with DMEM supplemented 10% FBS and antibiotics from 10^{-1} to 10^{-7} (from 50 to 0.00005 mg maca mL^{-1}). Then, 100 µL of each dilution was added to six-replicated wells of the 96-well microplate seed with the cell culture. DMEM solution was only added to the control wells. The microplates were incubated at 37 °C and 5% CO2 for 24 h. The inert microscopy (Olympus ix71, Tokyo, Japan) was used to observe and image the morphological changes of the cells for the cytotoxicity assay.

Cell staining and image analysis

The supernatants were discarded from the microplates and the wells were washed with PBS. 40 µL of trypan blue solution (0.4%) was added to each well and kept for 5 min Then, all wells were washed with PBS (Aung et al., 2019). Blue color dyed cells was considered as dead and were photographed at 20x (1300 x 1300 pixels,

96 DPI) for the monolayer area imagination and the measurement of cell mortality with ImageJ 1.53 software (National Institutes of Health, Bethesda, Maryland, USA) (Figure 1.).

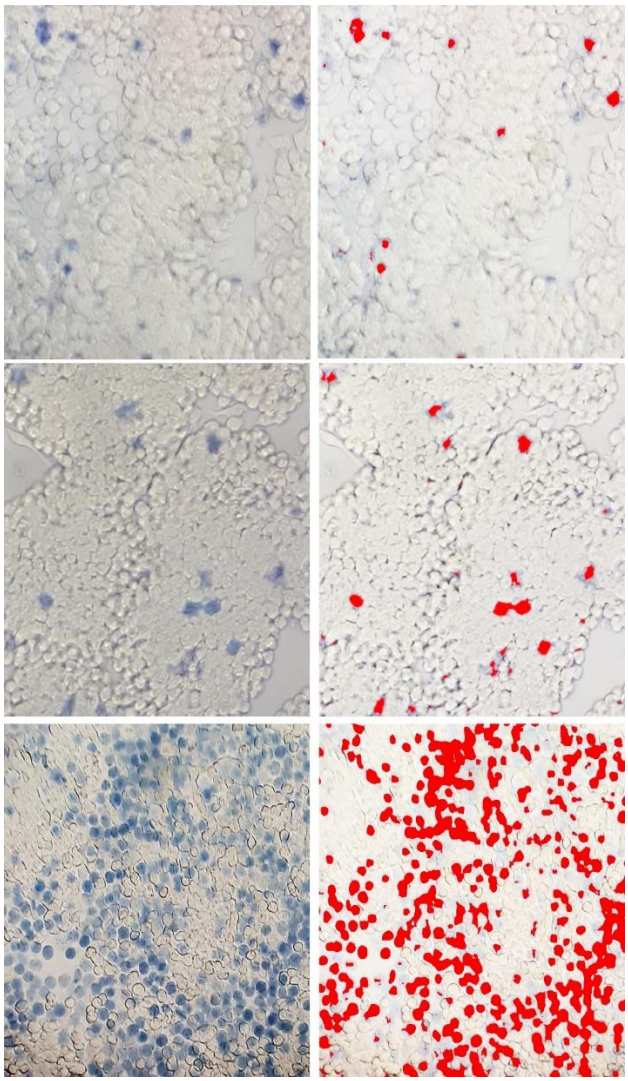

Figure 1. The dead cell counting (red coloured on right) and image analysis by ImageJ software.

Statistical analysis

The means of data and the standard deviations (SD) were calculated for each group using SPSS 21 software (SPSS Inc., IL, USA). The effects on cell viability were analysed by one-way analysis of variance (ANOVA). Differences among data means were compared using the Tukey post hoc test at a $P < 0.05$ level of significance.

RESULTS

The mean area of dead cells was determined as %0.76 of the total area in control wells and the mean dead cells were counted as 75.71 (±18.73) cells by the image analysis (Table 2.). And, no cytotoxic sign was observed in all six-replicate wells (0/6) of control (Table 1). For the aqueous solution of maca, the cytotoxic effect and the mean dead area of 2.45% was determined in the three of six-replicate wells (3/6) treated with the log-1 dilution (50 mg mL^{-1}) (Table 1. and 2.). But no cytotoxic effect was observed for the lower dilutions (from 10^{-2} to 10^{-7}) of the aqueous solution of maca (Table 1.). For the ethanol solution of maca, the mean dead areas of 29.17%, 25.66% and 22.51% were measured in the cells treated with the 10^{-1}, 10^{-2} and 10^{-3} dilutions (50, 5 and 0.5 mg mL^{-1}), respectively (Table 1. and 2.). These cytotoxic effects were observed in all (6/6), three (3/6) and one (1/6) of six-replicate wells for 10^{-1}, 10^{-2} and 10^{-3} dilutions respectively. However, there was no observed cytotoxic effect for the lower dilutions than the log-3 of the ethanol solution of maca (Table 1.).

When the results of cytotoxic dilutions of both solutions and the control were statistically compared with each other, the number of dead cells was significantly higher in the dilutions (331.5, 328.83 and 301.27 in the 10^{-1}, 10^{-2} and 10^{-3} respectively) of the ethanol solution than the aqueous solution and the cell control ($P<0,01$). Likewise, the dead cell count of the 10^{-1} (50 mg mL^{-1}) dilution of aqueous maca solution was statistically higher than the cell control ($P<0.01$)

Table 1. The cell mortality results of six-replicated wells on 96-well microplate.

	MW		ME	
Dilution (Log) (mg mL^{-1})	**Dead Area (%)**	**CPE of Wells**	**Death Area (%)**	**CPE of Wells**
10^{-1} (50 mg mL^{-1})	2.45	3/6	29.17	6/6
10^{-2} (5 mg mL^{-1})	0.95	0/6	25.66	3/6
10^{-3} (0.5 mg mL^{-1})	0.91	0/6	22.51	1/6
10^{-4} (0.05 mg mL^{-1})	0.71	0/6	0.97	0/6
-5 (0.005 mg mL^{-1})	0.56	0/6	0.91	0/6
-6 (0.0005 mg mL^{-1})	0.60	0/6	0.76	0/6
-7 (0.00005 mg mL^{-1})	0.71	0/6	0.81	0/6
Control	0.76	0/6	0.76	0/6

Table 2. The comparison of effects of the dilutions (10^{-1}-10^{-3}) on the cell viability by the image analysis of cell culture

Items	**Control**	**MW**	**ME**	**MW**	**ME**	**MW**	**ME**
		10^{-1} (50 mg **mL^{-1}**)		10^{-2} (5 mg **mL^{-1}**)		10^{-3} (0.5 mg **mL^{-1}**)	
Dead cell number (n)	75.71a	189b	331.5c	112.64a	328.83c	112.29a	301.27c
% of dead area (pixel^2)	0.76	2.45	29.17	0.95	25.66	0.91	22.51
SD	18.73	8.41	13.18	6.53	15.44	9.30	12.87
p value		<0.01		<0.01		<0.01	

DISCUSSION

Many compounds isolated from the plants, also maca, have been reported to possess potential cytotoxic activity (Bai et al., 2015; Zhou et al., 2017; 2018). Alkaloids and flavonoid, isolated from the aqueous acetone and ethanol extracts of maca powder, was reported as a cytotoxic compound on five human cancer cell lines at higher 40 µM (Bai et al., 2015; Zhou et al., 2017; 2018). Also, flavonoids in maca negatively affected the RAW 264.7 macrophages when compared the control cells (Bai et al., 2015). In the study of Del Valle Mendoza et al. (2014) the methanol extract of maca showed low cytotoxicity on MDCK cells, but the aqueous extract of maca powder did not show cytotoxic effect. Likewise, Wang et al. (2016) reported the immunomodulatory and antioxidant effects of aqueous polysaccharides from maca powder on RAW 264.7 macrophages without cytotoxic effect when compared the control. Ye et al. (2018) showed that the strong solvent (dichloromethane and ethyl acetate) extracts had serious cytotoxic effects. In the different solvent extracts of maca leaves, the aqueous extract showed more antioxidant capacity than chloroform, dichloromethane and chloroform-ethanolic extracts (Rodríguez-Huamán et al., 2017). In addition to the solvents, it was shown that the cytotoxic effects of maca extracts differed due to the kinds of cell cultures (Xia et al., 2018; Fu et al., 2021). In this study, the ethanolic dilutions of maca powder up to log 10^{-3} (50, 5 and 0.5 mg mL^{-1}) caused more cell mortality than the aqueous dilutions ($P<0.01$), similar to the results of Wang et al. (2016), Del Valle Mendoza et al. (2014) and Rodríguez-Huamán et al. (2017). However, the aqueous dilutions also increased the number of dead cells at only log 10^{-1} dilution (50 mg mL^{-1}) when compared with the control.

CONCLUSION

Many researchers reported that maca extracted by strong solvents such as alcohols adversely affected the cell viability, despite their rich bioactive compound content. Similarly, the alcoholic preparation of maca powder caused the

higher cytotoxic effect on the murine macrophage cells than the aqueous preparation in this study due to the dilution factor of the toxic solvent.

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Ethical approval: In the study, no experiments were made on any living thing or any personal information was used.

Conflict of interest: There is no conflict of interest.

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