Antiviral activity of macrophage-activating Chinese mixed herb hot water extract

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ABSTRACT
Aim: Macrophage-activating Chinese mixed herb (MACH) extract, a hot-water decoction of Cucurbita moschata (pumpkin seed), Carthamus tinctorius (safflower flower), P. asiatica (psyllium seed), and L. japonica (Japanese honeysuckle flower), is able to induce the production of interferon and interleukin, and hence reduce viral load in patients with hepatitis C virus (HCV) infection. The aim of this study was therefore to analyze the antiviral activity in the available models and identify the mechanism of alleviation of HCV infection.

Methods: The antiviral and anti-Herpes simplex virus (anti-HSV) activity of MACH extracts was examined using the plaque reduction assay in a mouse model of cutaneous HSV infection.

Results: MACH extracts inhibited plaque formation of HSV, Varicella zoster virus, cytomegalovirus, and poliovirus at 50% effective concentration for plaque formation (EC₅₀) of 3.09, 0.84, 1.54, and 3.3 mg/mL, respectively. MACH extract inhibited the late protein synthesis of HSV, indicating inhibition of viral DNA synthesis. MACH extract was given orally at 0, 40, and 400 mg in 200 μL water 7 days before infection in female BALB/c mice (6 weeks old) cutaneously infected with HSV. MACH extract was not toxic, as assessed by bodyweight changes, and significantly alleviated the development of skin lesions at days 6–8 (P < 0.05) but did not prolong the survival period.

Conclusion: On plaque reduction assay, MACH extract had significant but mild therapeutic anti-HSV activity. This anti-viral activity might be associated with the therapeutic antiviral activity in animals and humans.

KEY WORDS: antiviral activity, cytomegalovirus, herbal extract, Herpes simplex virus, poliovirus, Varicella zoster virus

INTRODUCTION
Herbal extracts have therapeutic anti-viral activities in animals, and the antiviral activity is due to antiviral compounds or activation of the immune response by cytokines or interferons [1–7]. Macrophage-activating Chinese mixed herb (MACH) extract is a hot-water decoction of Cucurbita moschata (pumpkin seed), Carthamus tinctorius (safflower flower), Plantago asiatica (psyllium seed), and Lonicera japonica (Japanese honeysuckle flower). Cucurbita moschata has an immune-enhancing effect via the production of T-helper (Th)1 cytokines (interleukin (IL)-2, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ) by activation of splenocytes and macrophages [8]. In animals, it induces the phagocytic activity of hemocytes in kuruma prawns [9].

MACH extract (EH0202) has been used in humans and had the following clinical efficacy in menopausal women and patients with hepatitis C virus (HCV) infection. After 32 post-menopausal women with menopausal complaints were given MACH extract (6 g/day for 6 months), there was a significant decrease in menopausal complaints (P < 0.0001), systolic (P < 0.001) and diastolic (P < 0.05) blood pressure, and facial skin surface blood flow (P < 0.05) [10]. Oral MACH extract significantly increased the percentage of good quality early stage blastocysts (P < 0.01) and significantly increased the percentage of late stage blastocysts in women [11]. MACH extract has been used in patients with chronic HCV infection. When MACH extract was given orally to 35 patients with chronic HCV for 3 months, a significant decrease in HCV-RNA (79%, P < 0.05) in patients with high viral titer and improvement in malaise, bloating sensation in the abdomen, and nausea and vomiting were reported. No serious adverse events were observed with EH0202 treatment [12]. Thus MACH extract has clinical benefits in patients with HCV infection.
The aim of this study was to elucidate the pharmacological basis of MACH extract in alleviating HCV viral load and improving the status of HCV infection, and to evaluate the direct antiviral activity of MACH extract against Herpes simplex virus (HSV), Varicella zoster virus (VZV), cytomegalovirus (CMV), DNA virus with envelope, and poliovirus 1 (PV), RNA virus without envelope, on plaque reduction assay in vitro, and the therapeutic antiviral activity in mice possibly due to cytokine response. In the present study MACH extract was found to have direct antiviral activity in vitro, and significant but mild therapeutic activity in HSV-infected mice, and this activity might contribute to reducing the HCV viral load, in addition to inducing IFN activity.

METHODS

Cells and viruses

Vero cells and human embryonic lung (HEL) cells were grown and maintained in Eagle’s minimum essential medium (MEM) supplemented with 5% calf serum and 10% fetal bovine serum (FBS), respectively.

The HSV strain used was HSV-1 (7401H) [2,13–15], and the other viruses used were VZV (Oka original strain), CMV [16,17], and PV (Sabin strain) [13]. HSV and PV were propagated in Vero cells. VZV and CMV were propagated in HEL cells. HSV, PV, and CMV were prepared from their infected cells by three cycles of freezing and thawing and stored at −80°C until use. VZV was stored as a cell-free virus in the sonication medium (phosphate-buffered saline containing 5% sucrose, 0.1% sodium glutamate, and 10% FBS) after sonication of infected cells and centrifugation [18–20]. Virus stocks were prepared from infected cultured cells and stored at −80°C until use. Mock antigen was prepared from uninfected cells.

Preparation

The MACH hot water extract was prepared using four components: Cucurbita moschata (pumpkin seed), Carthamus tinctorius (safflower flower), P. asiatica (psyllium seed), and L. japonica (Japanese honeysuckle flower). It was authenticated and purchased from Mikuni (Osaka, Japan), then heated with 15-fold the volume of water (heated to boiling for 1 h, then held for 30 min). The extract was concentrated under reduced pressure to give a soft extract, and the amount in terms of the original crude drugs was adjusted to 1:1.

Plaque reduction assay

The inhibitory activity of MACH extract on plaque formation by HSV-1, VZV, CMV, and PV was examined [1,2,13–15,21,22]. Duplicate culture of Vero cells in 60 mm plastic dishes was infected with 100 plaque-forming units (PFU)/0.2 mL HSV-1 or PV for 1 h at room temperature. HEL cells were used to determine the antiviral activity of VZV and CMV. Cells were overlaid with 5 mL nutrient agarose (1%) medium containing various concentrations of the MACH extracts and then cultured at 37°C for 2 days (PV), 3 days (HSV-1), 4 days (VZV) or 8 days (CMV). The infected cells were fixed with 5% formalin solution and stained with 0.03% methylene blue solution. The number of plaques was counted under a dissecting microscope. The 50% effective concentration for plaque formation (EC50) was defined as the concentration at which the plaque number decreased to half of that in cells cultured without MACH extract. EC50 was determined from the curve relating the plaque formation (%) of the untreated culture to the concentration of the samples using the computer program Microplate Manager III (BioRad, Hercules, CA, USA).

Immunoprecipitation

The effect of MACH extract on viral protein (VP) synthesis in Vero cells infected with HSV-1 was examined. The cells were infected with HSV-1 and incubated in the presence of 0, 2, 5, or 7 mg/mL MACH extract at 37°C. HSV-1-infected and mock antigen-infected cells were labeled with 100 μCi/mL [35S]-methionine and [35S]-cysteine (37 TBq/mmol; GE Healthcare Bio-Science, Piscataway, NJ, USA) for 1–8 h after infection in the absence or presence of MACH extract. The labeled cells were lysed by sonication in RIPA buffer (20 mmol/L Tris, pH 8.0, 100 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecylsulfate (SDS)) and centrifuged at 100,000 ×g for 30 min at 4°C. The labeled VP were immunoprecipitated with immunoglobulin for human use containing an anti-HSV antibody produced in guinea pigs. Immune complexes were analyzed on SDS–polyacrylamide gel electrophoresis (SDS-PAGE) using 8% acrylamide gel, followed by fluorography.

Mouse model of cutaneous HSV-1 infection

The therapeutic activity of MACH extract in mice was examined as described previously [1,2,13–15,21]. Female BALB/c mice (6 weeks old) were purchased from Sankyo Labo Service, Tokyo, Japan. The right midflank of each mouse was clipped and depilated with a chemical depilatory, Hair Remover (Shiseido, Tokyo, Japan). One or 2 days later, the naked skin was scratched using a 27 G needle and 7 μL HSV-1 (7401H strain) suspension containing 1 × 106 PFU was applied to the scratched area. Delayed development of skin lesions was then assessed using the mouse HSV skin infection model.

MACH extract was given to mice through a cannula to ensure the dosage of 200 μL/mouse/day from 1 week before infection, and was continued after HSV skin infection in three groups of mice: (i) control group (n = 15); (ii) 40 mg/dose/day MACH extract (n = 15); and 400 mg/dose/day MACH extract (n = 15) until all mice died. HSV skin lesion...
Score and mortality were assessed twice daily until all mice died. Skin lesion score was defined as follows: 0, no lesion; 2, vesicles in the local region; 4, erosion and/or ulceration in the local region; 6, mild zosteriform lesion; 8, moderate zosteriform lesion; 10, severe zosteriform lesion and death.

Serum TNF-α in the infected mice (n = 5 from each of the three groups) was evaluated on days 0, 3, and 5 after infection using Quantikine Mouse TNF-α (R&D Systems, Minneapolis, MN, USA).

We conducted all procedures in conformance with the National Institute of Health Guide for the Care and Use of Laboratory Animals with the approval of the Animal Care Committee at the University of Toyama.

**Statistical analysis**

The therapeutic effects of MACH extract on the progress of skin lesions were examined in control mice and mice treated with 40 and 400 mg/day MACH extract for 6–8 days after infection using overlapping measurement analysis of variance. Survival time was assessed on log-rank test, and survival rate, the Kaplan–Meier method. $P < 0.05$ was defined as statistically significant.

![Figure 1](image1.png)

**Figure 1** | Plaque formation versus macrophage-activating Chinese mixed herb (MACH) extract concentration for (a) poliovirus (PV), (b) Herpes simples virus 1 (HSV-1), (c) Varicella zoster virus (VZV) and (d) cytomegalovirus (CMV). Plaque formation without MACH extract was defined as 100%. The 50% effective concentration for plaque formation (EC50) calculated from each profile was 3.09, 0.84, 1.54, and 3.3 mg/mL, for HSV, VZV, CMV, and PV, respectively. (The EC50 were therefore obtained from these four profiles and not from the mean profile of four plaque reduction assays.)

![Figure 2](image2.png)

**Figure 2** | Effect of macrophage-activating Chinese mixed herb (MACH) extract on plaque formation of cytomegalovirus (CMV). (a) No MACH extract (10 plaques); (b) 0.5 mg/mL MACH extract (four plaques); (c) 5 mg/mL MACH extract (0 plaques). (c) The morphology of human embryonic lung (HEL) cells was not changed by 5 mg/mL MACH extract, indicating no apparent cytotoxicity toward HEL cells.
RESULTS

Antiviral activity on plaque reduction assay
MACH extract had antiviral activity on the plaque reduction method for HSV, VZV, CMV, and PV (Fig. 1). The EC_{50} of MACH extracts for HSV, VZV, CMV, and PV was 3.09 ± 0.39, 0.84 ± 0.01, 1.54 ± 0.73, and 3.3 ± 1.12 mg/mL (n = 4), respectively. This indicates that MACH extract directly inhibited plaque formation, and that EC_{50} differed, indicating differences in viral susceptibility to components in the extract.

MACH extract reduced CMV plaque size depending on concentration (Fig. 2), in addition to reducing the number of CMV plaques. CMV proliferation (plaque formation) in HEL cells was inhibited by MACH extract. In the absence of MACH extract, 10 plaques were observed in this field, but only four were observed in the medium containing 0.5 mg/mL MACH extract. Plaque size also decreased. In the presence of 5 mg/mL MACH extract, no plaque and no change in cell morphology were observed. Based on this, MACH extract specifically inhibited the growth of CMV at a non-cytotoxic concentration.

HSV protein synthesis
Figure 3 shows the effects of MACH extract on HSV protein synthesis, using immunoprecipitation with anti-HSV serum, followed by SDS-PAGE. The five proteins, corresponding to VP4, VP12, VP13, VP22, and VP23, based on molecular weight and relative molar ratios of VP [23,24], were specifically decreased by MACH extract. These VP are virion components, late VP synthesized after viral DNA synthesis, while the other proteins were not affected by MACH extract, indicating the specificity of the MACH extract for viral late protein synthesis. Thus, the anti-HSV activity of MACH extract was characterized by plaque reduction assay and inhibition of viral late protein synthesis.

Anti-HSV activity in skin infection mouse model
MACH extract was given every day for 7 days before, and then every day after HSV infection until the mice died. There was no significant difference in mouse weight, indicating no apparent toxicity at the doses used in this study. Mice given MACH extract had a significant delay in skin lesion development (therapeutic effect) in the HSV skin infection mouse model (P < 0.05; Fig. 4). There was no significant difference in survival time or mortality rate. The MACH extract showed anti-HSV activity, but survival time and mortality were not improved. Thus, the MACH extract had anti-HSV activity in vitro and therapeutic anti-HSV activity in vivo, indicating that anti-HSV activity in vitro contributed to the therapeutic activity in HSV-infected mice.

We evaluated the effects of MACH extract on the induction of TNF-α in the serum of HSV-infected mice on days 0, 3, and 5 after infection: TNF-α was detected only on day 5 (Fig. 5). TNF-α level was 20.7 ± 36.9, 5.4 ± 12.1, and 63.4 ± 61.0 pg/mL in the control, 40 mg/day, and 400 mg/day groups, respectively (P = 0.12), indicating no association between TNF-α and therapeutic activity in HSV-infected mice.

DISCUSSION
MACH extract is a hot-water decoction of Cucurbita moschata (pumpkin seed), Carthamus tinctorius (safflower flower), P. asiatica (psyllium seed), and L. japonica

Figure 3 | Effect of macrophage-activating Chinese mixed herb (MACH) extract on Herpes simplex virus (HSV) protein synthesis. Mock, mock antigen-infected cells; arrows, HSV proteins specifically decreased by MACH extract; the other proteins were not affected by increase in MACH extract concentration, indicating the specificity of MACH extract for viral protein synthesis of these five proteins. Open arrows, molecular weight (MW).
Japanese honeysuckle flower. *Cucurbita moschata* has an immune-enhancing effect through the production of Th1 cytokines (IL-2, TNF-α, and IFN-γ) by activation of splenocytes and macrophages [25]. Catechins from various plants have an antiviral effect after injection but do not show any effect after oral treatment [26]. Thus, the activity *in vitro* does not always reflect the therapeutic activity in animals and humans.

*Cucurbita moschata* induces IFN and cytokine production [8] and was added to MACH extract with the aim of enhancing the regulation of living organisms by combining herbal medicines that have historically been used safely [9–12,27]. MACH extract was effective in reducing the mortality rate of *Marsupenaeus japonicus* (kuruma shrimp) after a challenge with White spot syndrome virus (WSSA) [9]. That study clearly demonstrated that feeding kuruma shrimp with MACH extract increased total hemocyte count, prophe- nol oxidase, and phagocytic activity, thus resulting in protection against WSSV infection. In addition, MACH extract significantly reduced HCV-RNA in patients with high viral titer after 3 months of treatment, with no serious adverse events [12].

MACH extract inhibited plaque formation of HSV, VZV, CMV and PV with varying EC₅₀ concentration, ranging from 0.84 to 3.3 mg/mL. HSV, VZV, and CMV possess similar replication machinery, but have similar antiviral spectra and different EC₅₀ with regard to acyclovir and ganciclovir. MACH extract inhibited late protein synthesis of HSV, indicating that it inhibited viral DNA synthesis. There was a significant delay in the development of skin lesions in a mouse HSV cutaneous infection model during days 6–8, but bodyweight was not influenced by the MACH extracts, indicating that the therapeutic activity was not due to toxicity. Antiviral activity was not observed in the early phase of infection but was in the late phase, suggesting that the therapeutic activity would be due to augmentation of the host defense response, including cytokines, as observed [9–12,27]. This suggests that antiviral compound(s) in the MACH extracts might be absorbed and have therapeutic activity in HSV-infected mice.

We evaluated the effects of MACH extract on the induction of TNF-α in the serum of HSV-infected mice on days 0, 3, and 5 after infection, and TNF-α was detected only on day 5 (Fig. 5). There was no significant difference between treatment groups (*P = 0.12*). The local reaction of infected skin is important in the recovery from HSV infection in a cutaneous infection model [6], and no effect of MACH extract on the induction of TNF-α in the serum of HSV-infected mice treated with (■) no macrophage-activating Chinese mixed herb (MACH) extract (control); (■) 40 mg/day MACH extract and (■) 400 mg/day MACH extract. TNF-α was detected only on day 5 after infection (*P = n.s. between groups; n = 5).
extract on TNF-α was observed with regard to treatment effect in this system.

In this study, the antiviral activity of MACH extract was reflected in the inhibition of not only enveloped DNA virus but also RNA virus without an envelope at the cell culture level, in the inhibition of plaque formation. Furthermore, MACH extract had therapeutic anti-HSV activity in HSV-infected mice. This suggests that the antiviral activity in vitro might be associated with the therapeutic activity in an animal model of HSV infection, and that this activity may be closely related to the clinical observation of lower viral load in patients with chronic HCV infection [12]. As well as showing clinical efficacy in patients with HCV, improvement in fertility markers and vasomotor response has also been observed in post-menopausal women [10–12,27]. Thus, this study has demonstrated the antiviral activity in vitro and therapeutic anti-HSV activity in vivo of MACH extract, and provides a scientific basis for one of the uses of MACH extract.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Kurokawa M, Basnet P, Ohsugi M et al. Anti-herpes simplex virus activity of moronic acid purified from Rhus javanica in vitro and in vivo. J. Pharmacol. Exp. Ther. 1999; 289: 72–78.
2. Kurokawa M, Hozumi T, Basnet P et al. Purification and characterization of eugenin as an anti-herpesvirus compound from Geum japonicum and Syzygium aromaticum. J. Pharmacol. Exp. Ther. 1998; 284: 728–735.
3. Kurokawa M, Ohaya H, Hozumi T, Namba T, Nakano M, Shiraki K. Assay for antiviral activity of herbal extracts using their absorbed sera. Chem. Pharm. Bull. (Tokyo) 1996; 44: 1270–1272.
4. Kurokawa M, Tsuruta M, Brown J, Fukuda Y, Shiraki K. Effect of interleukin-12 level augmented by Kakkon-to, a herbal medicine, on the early stage of influenza infection in mice. Antiviral Res. 2002; 56: 183–188.
5. Kurokawa M, Yamamura J, Li Z et al. Antipyretic activity of ginglyo-san, a traditional medicine, in influenza virus-infected mice. Chem. Pharm. Bull. (Tokyo) 1998; 46: 1444–1447.
6. Nagasaka K, Kurokawa M, Imakita M, Terasawa K, Shiraki K. Efficacy of kakkon-to, a traditional herb medicine, in herpes simplex virus type 1 infection in mice. J. Med. Virol. 1995; 46: 28–34.
7. Lipipun V, Kurokawa M, Suttisri R et al. Efficacy of Thai medicinal plant extracts against herpes simplex virus type 1 infection in vitro and in vivo. Antiviral Res. 2003; 60: 175–180.
8. Kim HY, Nam SY, Yang SY, Kim HM, Jeong HJ. Cucurbita moschata Duch. and its active component, betacarotene effectively promote the immune responses through the activation of splenocytes and macrophages. Immunopharmacol. Immunotoxicol. 2016; 38: 319–326.
9. El Asely AM, Shaheen AA, Abbass AA et al. Immunomodulatory effect of plant-mixed feed in kuruma shrimp, Marsupenaeus japonicus, and its protective efficacy against white spot syndrome virus infection. J. Fish Dis. 2010; 33: 859–863.
10. Usihoryama T, Yoshida S, Tadaki K, Ikeda A, Ueki M. Clinical efficacy of EH0202, a Kampo formula, on the health of middle-aged women. Am. J. Chin. Med. 2004; 32: 755–770.
11. Usihoryama T, Yokoyama N, Hakukawa M, Sakuma K, Ichikawa F, Yoshida S. Clinical efficacy of macrophage-activating Chinese mixed herbs (MACH) in improvement of embryo qualities in women with long-term infertility of unknown etiology. Am. J. Chin. Med. 2012; 40: 1–10.
12. Kaji K, Yoshida S, Nagata N et al. An open-label study of administration of EH0202, a health-food additive, to patients with chronic hepatitis C. J. Gastroenterol. 2004; 39: 873–878.
13. Kurokawa M, Ochiai H, Nagasaka K et al. Antiviral traditional medicines against herpes simplex virus (HSV-1), poliovirus, and measles virus in vitro and their therapeutic efficacies for HSV-1 infection in mice. Antiviral Res. 1993; 22: 175–188.
14. Yoshida Y, Li Z, Kurokawa M, Kawana T, Imakita M, Shiraki K. Growth of herpes simplex virus in epidermal keratinocytes determines cutaneous pathogenicity in mice. J. Med. Virol. 2005; 75: 421–426.
15. Kurokawa M, Hozumi T, Tsuruta M, Kadota S, Namba T, Shiraki K. Biological characterization of eugenin as an anti-herpes simplex virus type 1 compound in vitro and in vivo. J. Pharmacol. Exp. Ther. 2001; 297: 372–379.
16. Kuramoto T, Daikoku T, Yoshida Y et al. Novel anticytomegalovirus activity of immunosuppressant mizoribine and its synergism with ganciclovir. J. Pharmacol. Exp. Ther. 2010; 333: 816–821.
17. Oshima K, Kanda Y, Kako S et al. Case report: Persistent cytomegalovirus (CMV) infection after haploidentical hematopoietic stem cell transplantation using in vivo alemtuzumab: Emergence of resistant CMV due to mutations in the UL97 and ULS4 genes. J. Med. Virol. 2008; 80: 1769–1775.
18. Shiraki K, Daikoku T, Takemoto M et al. Neutralizing anti-gH antibody of Varicella-zoster virus modulates...
distribution of gH and induces gene regulation, mimicking latency. *J. Virol.* 2011; **85**: 8172–8180.

19. Shiraki K, Yoshida Y, Asano Y, Yamanishi K, Takahashi M. Pathogenetic tropism of varicella-zoster virus to primary human hepatocytes and attenuating tropism of Oka varicella vaccine strain to neonatal dermal fibroblasts. *J. Infect. Dis.* 2003; **188**: 1875–1877.

20. Shiraki K, Hayakawa Y, Mori H et al. Development of immunogenic recombinant Oka varicella vaccine expressing hepatitis B virus surface antigen. *J. Gen. Virol.* 1991; **72**: 1393–1399.

21. Kurokawa M, Nakano M, Ohyama H et al. Prophylactic efficacy of traditional herbal medicines against recurrent herpes simplex virus type 1 infection from latently infected ganglia in mice. *J. Dermatol. Sci.* 1997; **14**: 76–84.

22. Okuda T, Kurokawa M, Matsuo K, Honda M, Niimura M, Shiraki K. Suppression of generation and replication of acyclovir-resistant herpes simplex virus by a sensitive virus. *J. Med. Virol.* 2004; **72**: 112–120.

23. Honess RW, Roizman B. Proteins specified by herpes simplex virus. XI. Identification and relative molar rates of synthesis of structural and nonstructural herpes virus polypeptides in the infected cell. *J. Virol.* 1973; **12**: 1347–1365.

24. Honess RW, Roizman B. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. *J. Virol.* 1974; **14**: 8–19.

25. Kim YK, Mbonye U, Hokello J, Karn J. T-cell receptor signaling enhances transcriptional elongation from latent HIV proviruses by activating P-TEFb through an ERK-dependent pathway. *J. Mol. Biol.* 2011; **410**: 896–916.

26. Daikoku T, Horiba K, Miyata K et al. Polyphenols including catechin from green tea with in vitro antiviral activity exhibited anti-herpes simplex virus activity but not anti-influenza virus activity in mice. *J. Trad. Med.* 2011; **28**: 63–72.

27. Ushiroyama T, Yoshida S, Tadaki K, Ikeda A, Ueki M. A pilot study of a Kampo formula, EH0202, with intriguing results for menopausal symptoms. *J. Altern. Complement. Med.* 2004; **10**: 397–399.