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An ethanol extract of *Grifola frondosa* (Maitake mushroom) enhances the anti-angiogenic and anti-tumorigenic actions of polymethoxyflavonoid, nobiletin, *in vitro*

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Introduction

Flavonoids from medicinal plants possess pharmacological effects for preventing tumor progression by inhibiting tumor-cell proliferation, neoplastic angiogenesis, and tumor invasion. We reported that a citrus polymethoxyflavonoid, nobiletin (5,6,7,8,3',4'-hexamethoxy flavone) (Fig.1), exhibits antitumor-invasive and antitumorigenic actions *in vivo* and *in vitro*. In addition, water extract of *Grifola frondosa* (Maitake mushroom) has been reported to possess antitumor effects by enhancing the immune system. However, the anti-angiogenic actions of nobiletin and Maitake mushroom extract remain unclear. In the present study, we examined whether nobiletin and an ethanol extract of the Maitake mushroom (Maitake extract), which is termed Gripin® (Fig. 2), influenced the proliferation of human melanoma Mewo cells and the tube formation of human microvascular endothelial cells (HMVEC).

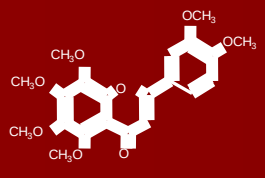


Fig. 1 Structures of nobiletin

Fig. 2 Ethanol extract of *Grifola frondosa* (Maitake) fruiting body (Gripin®)
A, *Grifola frondosa* (Maitake); B, dry powder of Maitake; and C, ethanol extract of Maitake, designated Gripin®.

Results

1. Nobiletin dose-dependently inhibited tube formation of HMVEC, whereas Maitake extract did not show any effect on the formation (Fig. 3). In addition, neither nobiletin nor Maitake extract showed cytotoxicity against HMVEC at concentrations tested (data not shown).
2. When HMVEC were co-treated with both nobiletin and Maitake extract, the nobiletin-mediated inhibition of tube formation was enhanced (Fig. 3).
3. Nobiletin slightly but significantly inhibited the proliferation of human melanoma Mewo cells in a dose-dependent manner, whereas the effect of Maitake extract on melanoma cell growth was negligible (Fig. 4).
4. The combination of nobiletin and Maitake extract synergistically inhibited tumor cell proliferation (Fig. 4).

Materials & Methods

Angiogenesis analysis. Human microvascular endotherial cells (HMVEC) (Kurabo, Osaka, Japan) were seeded on ECMatrix™ (Millipore, MA, USA), which was solidified at 37 °C prior to seeding, and then treated with nobiletin (8-32 μM) (Shaanxi Huike Botanical Development, Shaanxi Province, China) and/or maitake extract (100-400 μg/ml) (Fig. 2) for 16 h. After the treatments, the formation of cellular networks (tube formation) was inspected under an inverted light microscope at x40 magnification. Quantification of tube formation was performed by pattern recognition (values: 0-5), of which numerical value is associated with a degree of angiogenesis progression, according to the manufacturer's instruction (Table 1). Cellular networks chosen randomly five areas per well were photographed, and then the values averaged.

Cell proliferation analysis. Human melanoma Mewo cells (1 x 10^4 cells/well) were seeded onto 96-well multiplate and cultured for 24 h to achieve the complete adhesion. The cells were treated with nobiletin (8-32 μM) in the presence or absence of Maitake extract (400 μg/ml) for another 24 h, and then Alamar blue reagent (Biosource International, CA, USA) was incubated for the last 3 h of treatment. The fluorescence of incorporated Alamar blue was measure with excitation at 570 nm and emission at 600 nm according to the manufacturer's instruction.

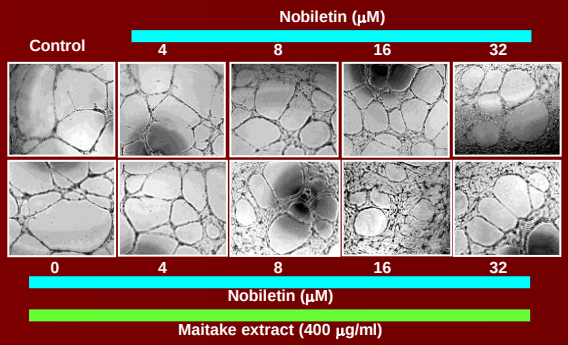
Statistical analysis. Data are presented as mean ± standard deviation (SD), and were analyzed by a one-way analysis of variance (ANOVA) and by the Fisher test for multiple comparisons. A value of $p<0.05$ was considered to indicate a statistically significant difference.

Table 1 A numerical value of angiogenesis progression

Pattern	Value
Individual cells, well separated	0
Cells begin to migrate and align themselves	1
Capillary tubes visible. No sprouting	2
Sprouting of new capillary tubes visible	3
Closed polygons begin to form	4
Complex mesh like structures develop	5

Conclusion

These results suggest that nobiletin exhibits an anti-angiogenic effect by inhibiting the tube formation of vascular endothelial cells. Moreover, the combination of Maitake extract and nobiletin is likely to be a novel clinical strategy for cancer therapy against melanoma to prevent both angiogenesis and tumorigenesis.



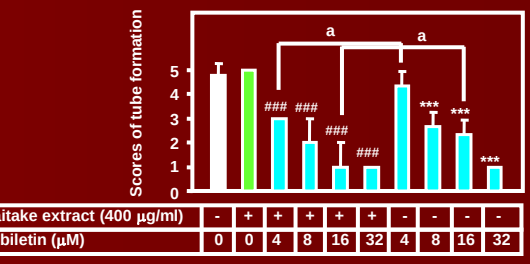


Fig. 3 Maitake extract enhances the anti-angiogenic activity of nobiletin against tube formation of HMVEC
A: HMVEC on 96-well multiplate coated with ECMatrix™ were treated with nobiletin (4-32 μM) in the presence or absence of Maitake extract (400 μg/ml) for 16 h. B: A numerical value (0-5) associated with a degree of tube formation was assessed as described in Materials and Methods. Data are shown as mean ± SD of three individual wells. ***, significantly different from untreated cells (Control) ($p<0.001$). ###, significantly different from Maitake extract-treated cells ($p<0.001$). a, significantly different from nobiletin-treated cells at each concentration ($p<0.05$).

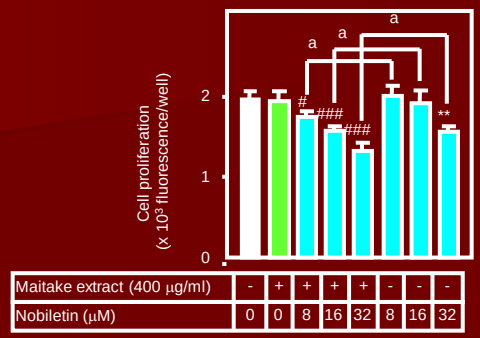


Fig. 4 Enhancement of nobiletin-induced antitumor proliferation by Maitake extract in human melanoma cells
Human melanoma Mewo cells in 96-well multiplates were treated with nobiletin (4-32 μM) in the presence or absence of Maitake extract (400 μg/ml) for 24 h. After the treatments, Alamar blue assay was performed to measure cell proliferation. Data are shown as mean ± SD of five wells. **, significantly different from untreated cells ($p<0.01$). # and ###, significantly different from Maitake extract-treated cells ($p<0.05$ and 0.001, respectively). a, significantly different from nobiletin-treated cells at each concentration ($p<0.05$).