Inhibitory Effect of Citrus -Hydroxy-3,6,7,8,3′,4′- hexamethoxyflavone on Phorbol Ester-induced Skin Inflammation and Tumor Promotion in Mice
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Polymethoxyflavones (PMFs), which are found exclusively in the Citrus genus, particularly in the peel of sweet oranges (Citrus sinensis) and mandarin oranges (Citrus reticulate), have a broad spectrum of biological activity including anticarcinogenic, anti-inflammatory, and antitumor activities. The intake of citrus fruit has been suggested to prevent the development of certain human cancers. It is also commonly recognized that cancer induction can be prevented by ingestion of certain food phytochemicals, and flavonoids in Citrus fruits and juices are one of the most prominent cancer-preventing agents. Compared to polyhydroxylated flavonoids, PMFs have higher permeability through the small intestine and are readily absorbed into the human blood circulatory system. The recent isolation of 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (5-OH-HxMF) from sweet orange peel extract and the reported biological activities of other PMFs promoted us to study its anti-inflammatory and anti-tumor activities. We have estimated that the amount of 5-OH-HxMF in dried orange peel is about 10 ppm (10 mg/kg of dried orange peel).

It has been known that inflammation is causally linked to carcinogenesis and acts as a driving force in premalignant and malignant transformation of cells. Topical application of 12-0-tetradecanoylphorbol 13-acetate (TPA) to 7,12- dimethylben[α]anthracene (DMBA)-initiated mice leads to edema and papilloma formation by enhancing inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein expression. Specific iNOS and COX-2 inhibitors are able to counteract the biological events. Recent studies revealed that constitutive activation of STATs (signal transducers and activators of transcriptions), particularly STAT3, is found in a number of primary human epithelial tumors and cancer cell lines. Persistently active STAT3 induces tumor angiogenesis by up-regulation of vascular endothelial growth factor (VEGF) and its immune evasion. Activated nuclear factor-κB (NFκB) often facilitates transcription of numerous genes, including iNOS and COX-2, resulting in inflammation and tumorigenesis. Activation of NFκB by TPA is induced by a cascade of events leading to the activation of inhibitor κB (IκB) kinases (IKKs), which in turn phosphorylates IκB. The subsequent ubiquitination and proteasomal degradation of IκB lead NFκB free to translocate to the nucleus. These kinases can be activated through phosphorylation by upstream kinases, including NFκB-inducing kinase, mitogen-
activated protein kinase (MAPK), and protein kinase C. More importantly, iNOS has been shown to be involved in regulating COX-2, which plays a pivotal role in colon tumorigenesis. These observations suggest that iNOS may exacerbate tumorigenesis.

In this research, we have studied the effect of 5-OH-HxMF on the TPA-induced iNOS and COX-2 expression in mouse skin, explored underlying molecular mechanisms, tested the anti-inflammatory activity of 5-OH-HxMF in mouse skin following TPA application, and investigated the inhibitory effect of 5-OH-HxMF on mouse skin tumor promotion using a two-stage carcinogenesis model including tumor incidence, multiplicity, and volume. We found that the topical application of 5-OH-HxMF can effectively inhibit the transcriptional activation of iNOS and COX-2 mRNA and protein in mouse skin stimulated by TPA. Pretreatment with 5-OH-HxMF resulted in the reduction of TPA-induced nuclear translocation of NFκB subunit and DNA binding by blocking phosphorylation of IκBα and p65 and subsequent degradation of IκBα. In addition, 5-OH-HxMF can inhibit TPA-induced phosphorylation and nuclear translocation of the STAT3. Moreover, 5-OH-HxMF can suppress TPA-induced activation of extracellular signal-regulated kinase (ERK)1/2, p38 MAPK, and phosphatidylinositol 3-kinase (PI3K)/Akt, which are upstream of NFκB.

We also evaluated the in vivo anti-inflammatory and antitumor promoting properties of 5-OH-HxMF by measuring epidermal thickness, leukocyte infiltration, and the rate of proliferating cell nuclear antigen-stained cells after a topical TPA application to mouse skin and found that 5-OH-HxMF significantly inhibited TPA-induced mouse skin inflammation by decreasing inflammatory parameters. Furthermore, 5-OH-HxMF significantly inhibited DMBA/TPA-induced skin tumor formation by reducing the tumor incidence and tumor multiplicity of papillomas at 20 weeks. Therefore, all these results revealed for the first time that 5-OH-HxMF is an effective antitumor agent and its inhibitory effect is through the down-regulation of inflammatory iNOS and COX-2 gene expression in mouse skin, suggesting that 5-OH-HxMF is a novel functional agent capable of preventing inflammation-associated tumorigenesis.

![Chemical structure of 5-hydroxy-3, 6, 7, 8, 3', 4'-hexamethoxyflavone](image)

**Figure 1.** Chemical structure of 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone.
Figure 2. Inhibitory effects of 5-OH-HxMF on phorbol ester-induced iNOS and COX-2 protein expression. (a) Time course for iNOS and COX-2 protein expression on topical application of TPA in mouse skin. (b) Mice were treated topically with acetone or 5-OH-HxMF 30 min prior to 10 nmol TPA. The epidermal proteins were analyzed for iNOS and COX-2 by Western blotting analysis.