

Nobiletin and 5-demethylnobiletin ameliorate hypopharyngeal squamous cell carcinoma by suppressing TGF- β -mediated epithelial-mesenchymal transition

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Received June 7, 2024; Accepted October 2, 2024

DOI: 10.3892/ol.2025.14922

Abstract. Hypopharynx squamous cell carcinoma accounts for 5% of all diseases diagnosed in Mexico. It is associated with poor oral hygiene, alcohol consumption and tobacco use and is usually diagnosed at an advanced stage, with metastasis to the lymph nodes. Metastasis from primary tumors occurs via a complex process called epithelial-mesenchymal transition (EMT), in which epithelial cells gradually acquire characteristics of mesenchymal cells, enabling their spread. Flavonoids have anticancer effects. In the present study, the effects of the polymethoxyflavones nobiletin (Nob) and 5-demethylnobiletin (5-DMN) on transforming growth factor (TGF)- β 1-induced EMT in hypopharyngeal squamous cell carcinoma cells were evaluated. Either polymethoxyflavone alone inhibited cell proliferation and combined treatment had no synergistic effect. The two flavonoids inhibited EMT by reversing the effects of TGF- β on morphological changes, migration and the expression of the markers E-cadherin, N-cadherin, Slug and Snail. Thus, Nob and 5-DMN are potential candidates for use in the treatment of oral squamous cell carcinoma.

Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common type of cancer, with an incidence of ~377,713 cases worldwide, 62% of which occur in developing countries. Of OSCC cases, approximately one-third are recurrent. Metastasis, disease recurrence and drug resistance are key factors in the low (<60%) 5-year survival rate of patients with

OSCC and a delay in metastasis contributes to prolonged survival of patients with OSCC (1-5).

The epithelial-mesenchymal transition (EMT) plays a key role in the pathogenesis of OSCC, specifically its progression from fibrotic transformation to metastasis. Polarized, quiescent epithelial cells become mesenchymal cells, giving cancer cells an invasive phenotype (1,5,6). The most widely studied factor in EMT induction is transforming growth factor (TGF)- β , which plays an important role in promoting tumor invasion and metastasis. The downregulation of E-cadherin is a distinctive marker of EMT progression and the increased expression of transcription factors such as Twist, Snail, Slug and ZEB1 is related to the establishment of invasive tumors (7-10).

The consumption of a diet rich in vegetables, legumes and cereals is strongly associated with the decreased manifestation of degenerative disorders such as cancer. Flavonoids are phenolic compounds synthesized in the secondary metabolism of plants. They are involved in the protection of plants from biotic stress factors (such as insect, microorganism and herbivore attacks) and abiotic stresses (such as drought, rain, frost and high temperatures). Flavonoids form a very diverse group and are classified as flavones, flavonols, isoflavonoids, flavanones, chalcones and anthocyanidins (11).

Polymethoxyflavones are compounds present in the peels of citrus fruits such as *Citrus nobilis*, from which the name nobiletin (Nob; 5,6,7,8,30',4'-hexamethoxyflavone) is derived (Fig. 1), as well as tangerine (*Citrus tangerina*, which has the highest Nob content), bitter orange (*Citrus aurantium*) and mandarin orange (*Citrus reticulata*). Nob has been shown to have effects against several types of cancer, including breast (12-17), gastric (13), lung (14), liver (15) and bone (16) cancers and OSCC (18).

Nob is degraded to 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone (5-DMN) via autolysis (18) or conversion in gastric juice (19). Nob uptake occurs in phases I and II of metabolism and *in vivo*, Nob demethylation is carried out by cytochrome P450 (20). Several investigations have shown that Nob metabolites are more potent than Nob in HT-29 cells derived from colon adenocarcinoma (18,21,22); the half maximal inhibitory concentration (IC₅₀) of 5-DMN in these cells is 22 μ M, whereas that of Nob is 46.5 μ M. Researchers have concluded that the hydroxyl group at position 5 is involved in important molecular interactions in metabolic regulation (23).

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Key words: nobiletin, 5-demethylnobiletin, epithelial-mesenchymal transition, transforming growth factor β -1

Among the effects associated with Nob are cell cycle arrest via the mitogen-activated protein kinase and Akt pathways (24,25), the induction of apoptosis (26) and the suppression of cell proliferation (27). The antimetastatic activities of Nob include the regulation of matrix metalloproteinase 1-9 and tissue inhibitor of metalloproteinase 1 expression in human fibrosarcoma (28) and the inhibition of the TGF- β -induced migration and invasion of non-small cell lung cancer cells (29,30). Thus, Nob has a broad spectrum of mechanisms against cancer development. The present study demonstrated that Nob and 5-DMN inhibited cell proliferation and regulated the expression of N-cadherin, E-cadherin, Snail and Slug, suggesting their potential value in the treatment of OSCC.

Materials and methods

Reagents. Nob and 5-DMN were purchased from Merck. TGF- β was purchased from PeproTech, Inc. Actin, E-cadherin, N-cadherin, Slug and Snail were obtained from Santa Cruz Biotechnology, Inc.

Cell culture. FaDu cells derived from hypopharyngeal squamous cell carcinoma were obtained from the American Type Culture Collection and cultured by incubation in Dulbecco's modified Eagle's medium (MilliporeSigma) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 μ U/ml penicillin and 100 μ g/ml streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C. Nob and 5-DMN (<0.5% of the total volume) were dissolved in dimethyl sulfoxide (DMSO).

Cell viability assay. Cell viability was determined via a 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. The cells [1x10⁵/well, determined by a TC20 automated counter (Bio-Rad Laboratories, Inc.)] were seeded in 96-well plates at 37°C for 12 h. They were grown overnight and then treated with different doses of Nob and 5-DMN for 24, 48 and 72 h. Viability was measured after the addition of MTT (0.5 mg/ml) for 4 h. At the end of the assay, 150 μ l of DMSO was added to dissolve the formazan crystals. The plates were read at 570 nm via a Synergy device (BioTek; Agilent Technologies, Inc.). The assay was repeated five times.

Colony-forming assay. FaDu cells (200/ml) were cultured in six-well plates at 37°C for 12 h and treated with TGF- β (3 pM) for 24 h. Nob (50 μ M), 5-DMN (50 μ M), or Nob (50 μ M) plus 5-DMN (10 or 25 μ M) was then added and the cells were cultured under normal conditions (5% CO₂, 37°C) for 18 days. The medium was changed every 3 days. Colonies were fixed with 4% paraformaldehyde at 37°C, stained with Giemsa solution at 37°C for 30 min (MilliporeSigma) and then counted. The assay was performed three times.

Cell migration assays. The cell migration capacity was determined via wound healing and Transwell assays. FaDu cells were seeded in six-well plates and cultured to confluence. A 200- μ l sterile pipette tip was then used to scratch a vertical line, followed by washing twice in sterile phosphate-buffered saline to remove the debris. The cells in Dulbecco's modified Eagle's medium without fetal bovine serum were then treated

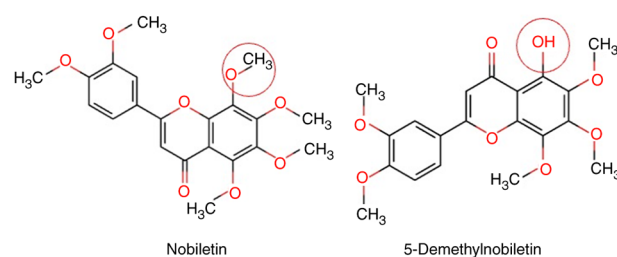


Figure 1. Chemical structures of nobiletin and 5-Demethylnobiletin.

for 24 or 48 h with DMSO vehicle (control), 3 pM TGF- β 1 + 50 μ M Nob, 50 μ M 5-DMN, or 50 μ M Nob + 10 μ M 5-DMN. A total of 15 images were obtained for each condition and the experiment was performed three times. For the wound healing assay, experiments were performed three times and images of each condition were captured with a Zeiss Primo-vert microscope (Zeiss AG). The data were analyzed with ImageJ 1.52a software (National Institutes of Health). The wound healing assay index was calculated using the following formula: Wound healing index=(X- Assay Group)/(X- Control Group)*100. For the Transwell assay, FaDu cells (n=10,000) were seeded in 24-well Transwell inserts (8-mm pore size) and treated with Nob or 5-DMN at different doses. After 24 h, the medium was removed and the cells were fixed with 4% paraformaldehyde at 37°C for 5 min. The membranes were then permeabilized with 100% methanol for 20 min and stained with thiazole orange (0.1 μ M) for 15 min at 37°C. The membranes were visualized under a Polyvar fluorescence microscope, (Leica GmbH) as previously described (17).

Western blotting. FaDu cells (1x10⁵) were seeded in six-well plates and treated with TGF- β (3 pM), + Nob (50 and 100 μ M) or (B) 5-DMN (50 and 100 μ M) and TGF- β (3 pM), + plus Nob (50 μ M), 5-DMN (50 μ M), Nob (50 μ M) + 5-DMN (50 μ M), or Nob (50 μ M) + 5-DMN (100 μ M) for 24 h. Lysates were prepared in 10 mM Tris-HCl, 0.15 mM NaCl, 1 M ethylenediaminetetraacetic acid, 20 mM NaF, 100 mM Na₃VO₄, 0.5% NP-40, 1% Triton, 1 mM phenylmethylsulfonyl fluoride (pH 6.8) and a protease inhibitor cocktail. The samples were centrifuged and the supernatants were recovered and stored at -80°C. Proteins were quantified via the Bradford assay and 40- μ g aliquots were separated via 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (110 V; 2 h). The proteins were transferred to polyvinylidene fluoride membranes, blocked with 5% skimmed milk at 37°C for 1 h and incubated with the following primary antibodies: Anti-E-cadherin (sc-71008) (1:10,000), anti-N-cadherin (sc-59987) (1:10,000), anti-Slug (sc-166476) (1:15,000), anti-Snail (sc-271977) (1:10,000) and anti-actin (sc-47778) (1:5,000; all from Santa Cruz Biotechnology, Inc.). The membranes were incubated overnight at 4°C, washed three times with washing buffer and incubated for 2 h with secondary antibody [anti-rabbit (sc-2004), anti-goat (sc-2354) immunoglobulin G horseradish peroxidase conjugate (1:10,000); Santa Cruz Biotechnology, Inc.]. The membranes were exposed to luminol reagent for 15 min (Santa Cruz Biotechnology, Inc.). The bands were analyzed with the ImageQuant LAS 500 program, software version 1.1.0. (General Electric, Healthcare Bio-Sciences AB Björkgatan 30).

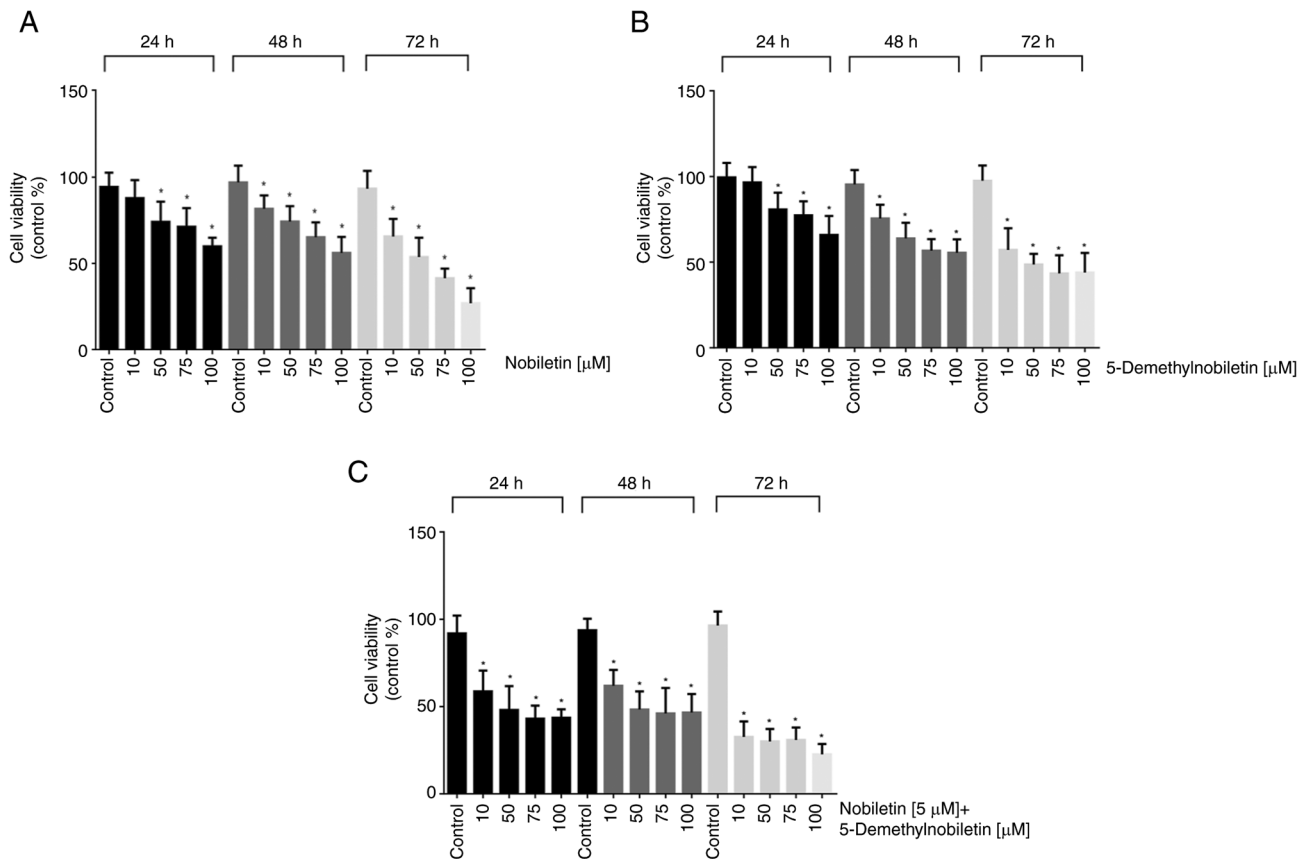


Figure 2. Nob and 5-DMN reduced FaDu cell viability, as determined by the MTT assay. The cells were treated with different concentrations of (A) Nob, (B) 5-DMN and (C) Nob plus 5-DMN for 24, 48 and 72 h. *P<0.05.

Data analysis. Experiments were performed three times in triplicate. Statistical analysis was performed with GraphPad Prism 5 (Dotmatics). Data were shown as mean ± standard deviation. Paired Student's t-test (two-way) was used for comparison between groups. Between-group comparisons were performed by one-way analysis of variance (ANOVA) followed by Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of Nob and 5-DMN on cell proliferation. Nob significantly inhibited the growth of FaDu cells in a dose- and time-dependent manner. The IC₅₀ values of Nob in FaDu cells at 24, 48 and 72 h were 31.81, 22.65 and 19.92 μM, respectively (Figs. 2A and 3A). 5-DMN treatment had a greater inhibitory effect, yielding IC₅₀ values of 42.03, 11.02 and 5.84 μM at 24, 48 and 72 h, respectively (Figs. 2B and 3B). In the combined treatment, the two agents had a synergistic cytotoxic effect, yielding IC₅₀ values of 7.13, 6.59 and 4.9 μM at 24, 48 and 72 h, respectively (Figs. 2C and 3C). The IC₅₀ values obtained at 24 h were used as the treatment doses in subsequent assays.

Nob and 5-DMN suppress TGF-β-induced cellular morphological changes. After 24 h of TGF-β1 treatment, the morphology of the cells changed from cuboidal (Fig. 4A) to an elongated spindle shape (Fig. 4B). The cells recovered

their cuboidal morphology when exposed to NOB (10 and 50 μM; Fig. 4C and D). Similar results were obtained with 5-DMN (10 and 50 μM) treatment, but this treatment induced cell detachment and clustering (Fig. 4E and F).

Effects of Nob and 5-DMN on cell migration. The results of the wound-healing assay differed significantly between the control and TGF-β1-treated cells, with no significant difference observed between Nob- and 5-DMN treated cells or between either of these groups and TGF-β1-treated cells. Combined treatment with Nob and 5-DMN yielded significant differences between the groups at 24 and 48 h (Fig. 5). In the Transwell assay, compared with TGF-β1, Nob significantly suppressed migration, but 5-DMN and the combination treatment did not (Fig. 6). These results suggested that Nob played an important role in cell migration.

Effects of Nob and 5-DMN on colony formation. In the colony formation assay, compared with the control, TGF-β1 treatment significantly increased colony formation. Nob and/or 5-DMN treatment significantly reversed the effect of TGF-β1, reducing the number of colonies formed and inhibiting cell survival (Fig. 7).

Nob and 5-DMN inhibit the effects of TGF-β1 on EMT. Nob and 5-DMN inhibited the expression of the EMT markers N-cadherin, Snail and Slug in FaDu cells incubated with TGF-β1 in a dose-dependent manner, subsequently

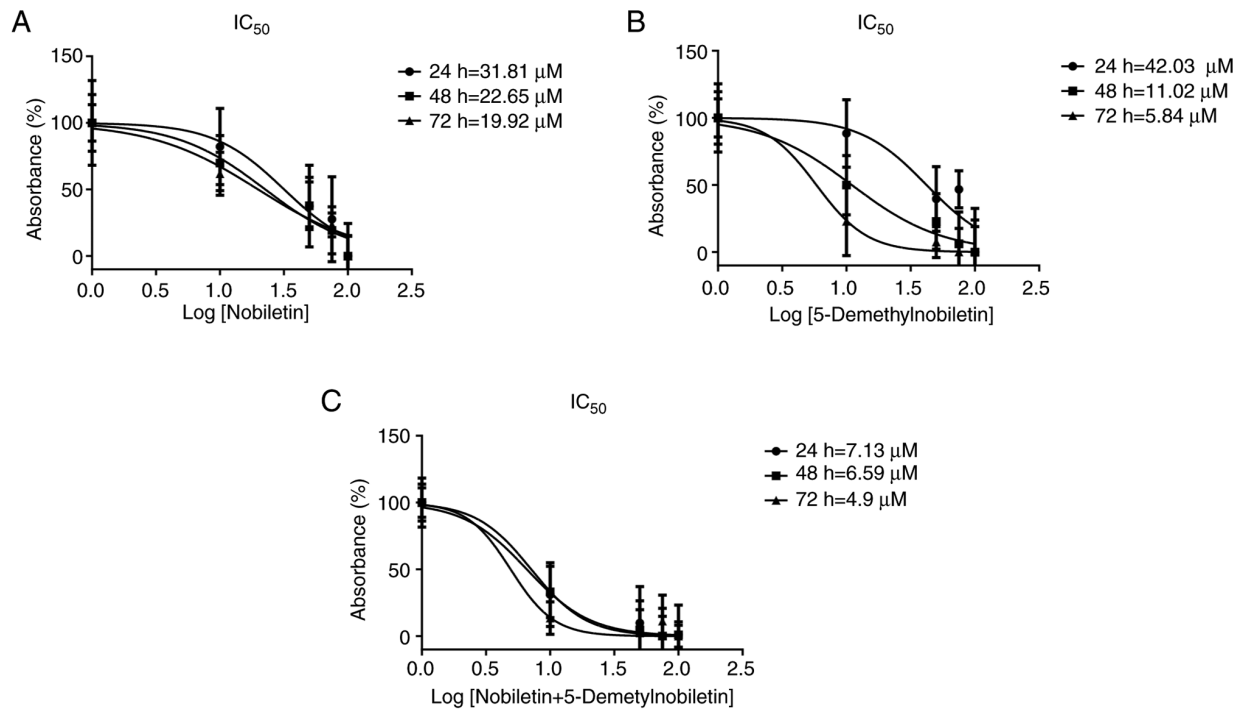


Figure 3. IC₅₀ in FaDu cells. IC₅₀ curves of (A) nobiletin, (B) 5-Demethylnobiletin and (C) both agents in FaDu cells after 24, 48 and 72 h. IC₅₀, half maximal inhibitory concentration.

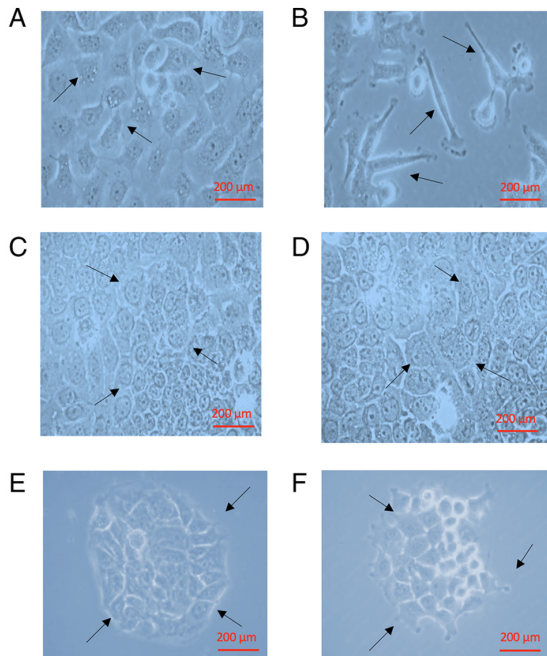


Figure 4. Effects of TGF on the morphological characteristics of FaDu cells. (A) Control, Dulbecco's modified Eagle's medium + 10% fetal bovine serum; (B) + TGF- β 1 (3 pM); (C) TGF- β 1 + Nob (10 μ M); (D) TGF- β 1 + Nob (50 μ M); (E) TGF- β 1 + 5-DMN (10 μ M); or (F) TGF- β 1 + 5-DMN (50 μ M). Scale bar, 200 μ m. TGF, transforming growth factor; Nob, nobiletin; 5-DMN, 5-Demethylnobiletin. The arrows represent the morphological changes of FaDu cells.

increasing E-cadherin expression (Fig. 8A). The combination treatment increased the expression of E-cadherin and decreased the expression of N-cadherin, Slug and Snail

(Fig. 8B). These data suggested that Nob and 5-DMN regulate FaDu cell proliferation, migration and invasion and that these agents combined have additional effects on EMT marker expression. In addition, Nob, 5-DMN and combination treatment induced the expression of Bax and suppressed the expression of Bcl2, highlighting the roles of these flavonoids in the regulation of apoptosis (Fig. 8B).

Discussion

Cancer imposes a high public health burden and has a high mortality rate. As cancer progresses, tumor cells undergo a number of changes in the expression of adhesion molecules, leading to their detachment from the basal layer and the acquisition of invasive phenotypic characteristics (that is, EMT). Epithelial cells acquire a mesenchymal architecture that allows them to invade new positions.

Hypopharyngeal squamous cell carcinoma is a common head and neck cancer with a relatively high incidence in men and is associated with alcohol consumption, tobacco use and *Papillomavirus* infection. When this type of cancer is diagnosed, it has typically already metastasized to the lymph nodes and lungs, resulting in a very low survival rate and major side effects that reduce the quality of life of patients (29).

The present study evaluated the effect of Nob and 5-DMN on EMT in FaDu cells derived from a hypopharyngeal squamous cell carcinoma. Nob has attracted increasing research interest because of its multiple benefits in the treatment of diseases such as diabetes (31), osteoporosis (32) and neurodegenerative diseases (33). Like other flavonoids, it has anti-inflammatory (34) and antioxidant (35) activities and hepatoprotective and cardioprotective (36) functions. These

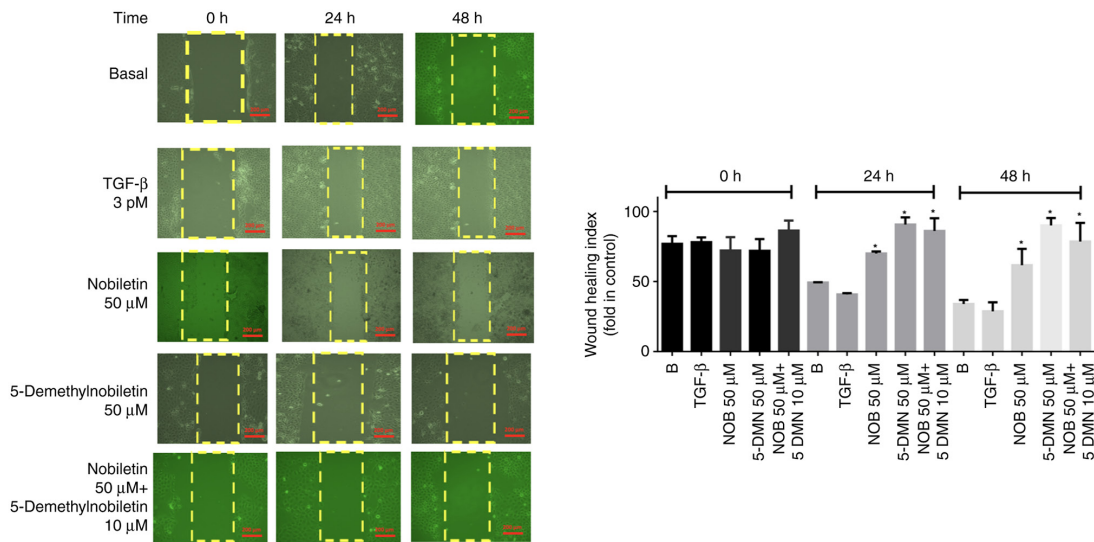


Figure 5. Effects of Nob, 5-DMN and both agents on the TGF-β1-induced migration of FaDu cells. *P<0.05. Scale bar, 200 μm. Nob, nobiletin; 5-DMN, 5-Demethylnobiletin; TGF, transforming growth factor.

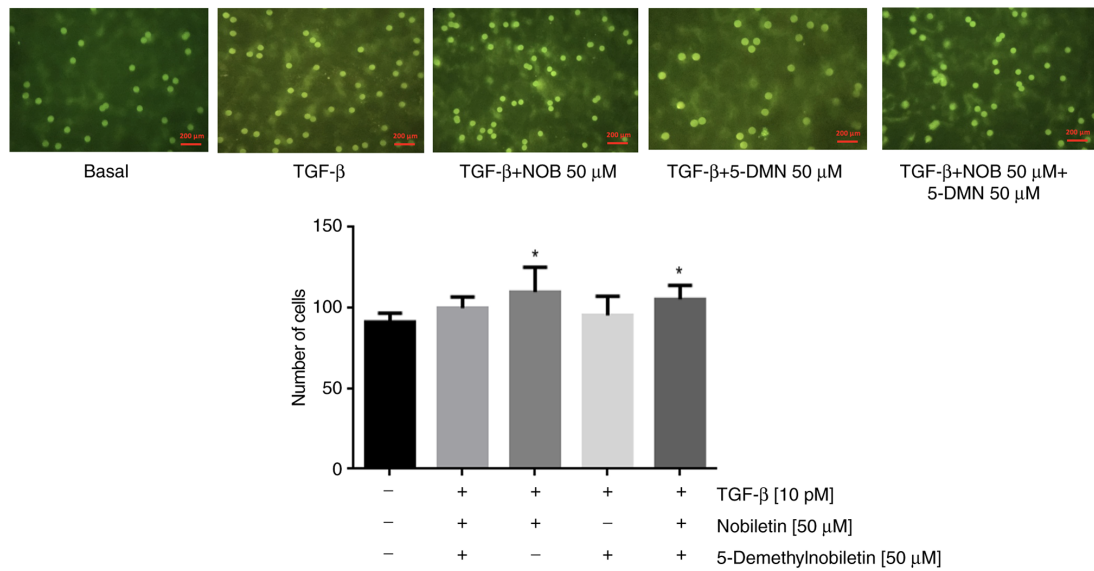


Figure 6. Effects of Nob, 5-DMN and Nob + 5-DMN on the TGF-β-induced migration of FaDu cells incubated in DMEM + 10% SBF with TGF-β, TGF-β + Nob (50 μM), TGF-β + 5-DMN (50 μM), or TGF-β + Nob (50 μM) + 5-DMN (50 μM). *P<0.05. Scale bar, 200 μm. Nob, nobiletin; 5-DMN, 5-Demethylnobiletin; TGF, transforming growth factor.

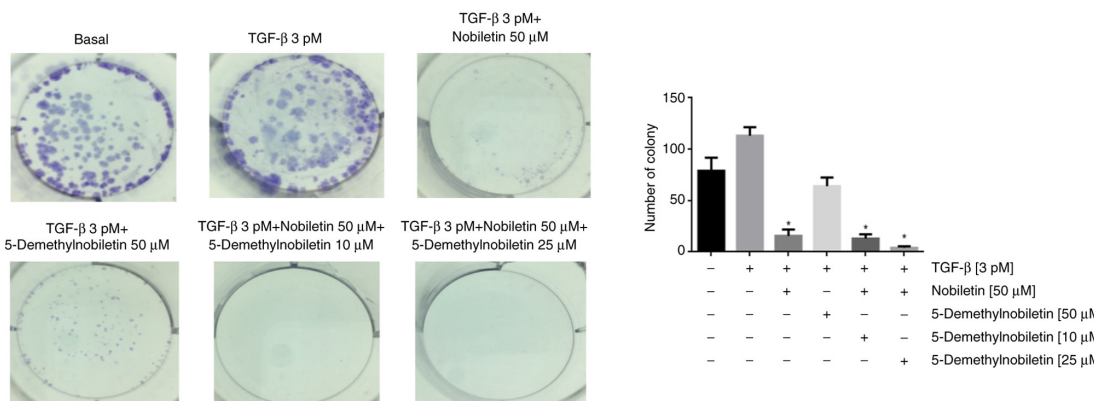


Figure 7. Colony formation of FaDu cells incubated with TGF-β1 and exposed to Nob, 5-DMN or Nob + 5-DMN. *P<0.05. TGF, transforming growth factor; Nob, nobiletin; 5-DMN, 5-Demethylnobiletin.

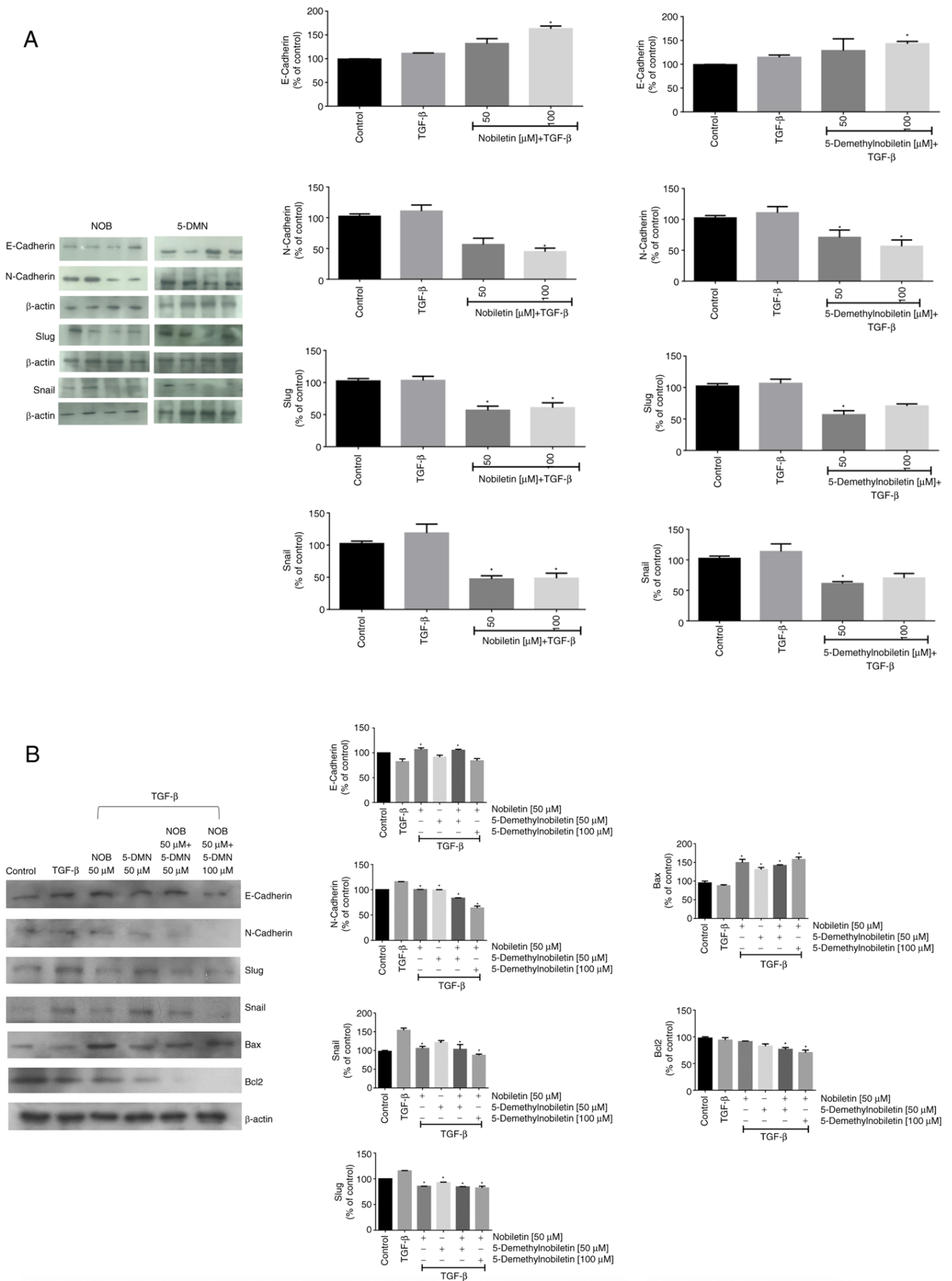


Figure 8. Expression levels of E-cadherin, N-cadherin, Slug and Snail. FaDu cells were treated with TGF- β (3 pM), + plus (A) Nob or (B) 5-DMN for 24 h. Actin was used as the loading control. *P<0.05. TGF, transforming growth factor; Nob, nobiletin; 5-DMN, 5-Demethylnobiletin.

flavones are obtained naturally from citrus peel and despite their broad spectrum of functions, some research indicates that 5-DMN has greater biological activity than the parent flavones (22,37,38).

As 5-DMN has shown greater potency in the inhibition of colon cancer growth (18), the present study compared the effects of Nob and 5-DMN on TGF- β 1-induced EMT in the FaDu cell line, which is derived from a squamous cell carcinoma of the hypopharynx. The present study is important because 5-DMN is extracted from citrus peel and is a metabolic product derived from Nob (39). In the present study, 5-DMN had greater potency than Nob in the inhibition of cell proliferation, but no significant difference in migration or the regulation of EMT marker expression was observed between the treatment groups.

5-DMN has been reported to inhibit colon cancer cell proliferation and induce apoptosis (23), with an IC_{50} of 42.03 μ M, similar to the value obtained in the present study at 24 h. In a lung cancer cell line, treatment with the synthetic form yielded an IC_{50} of 21.8 μ M and resulted in cell cycle regulation and the promotion of apoptosis (40). In the A459 cell line, Nob treatment yielded an IC_{50} of 100 μ M (41), which was greater than that reported for FaDu cells in the present study. Among the effects associated with 5-DMN are induction of apoptosis via a reduction of the mitochondrial membrane potential (in neuroblastoma) and cell cycle arrest (42). However, no study has examined the effect of 5-DMN on EMT to the best of the authors' knowledge. In lung adenocarcinoma cells, Nob decreases cell viability and inhibits TGF- β 1-induced EMT at a dose of 100 μ M and reduces Snail, Slug and Twist expression levels at a dose of 20 μ M. The present study obtained similar results for these doses. Similarly, TGF- β 1-stimulated colony formation was suppressed (29), in the FaDu cell line. Similar results have been reported for glioma cells treated with 15 μ M Nob (43).

The present study compared the effect of Nob and 5-DMN on EMT in an *in vitro* model. It was found that TGF- β -induced EMT was effectively blocked by both agents and the results are similar to those obtained with other flavonoids, such as biochanin A (44), silibinin (45) and scutellarin, in gastric cancer (46).

The results of the present study revealed that Nob and 5-DMN inhibited proliferation and cell migration. However, combined treatment with both agents did not result in a synergistic inhibitory effect. These results were similar to those reported with silibinin (45). The present study found that Nob and 5-DMN reversed the effects of TGF- β on the expression of EMT markers in a manner similar to that reported in renal cell carcinoma subjected to hypoxia (47).

The present study revealed that either agent alone or the combination of both agents inhibited EMT and altered the Bax/Bcl2 ratio. However, the study has limitations that need to be addressed in further investigations. First, it did not determine the mechanism of action for EMT suppression and apoptosis induction and, second, the role of migration and apoptosis needs to be assessed in an *in vivo* setting.

In conclusion, discrepancies in the effective concentrations of Nob and 5-DMN may be a function of differences in tissues and/or their sensitivity to flavonoids. Thus, further research on apoptosis mechanism and its role on xenograft mouse models

studies should be conducted to determine their therapeutic effectiveness.

Acknowledgements

Not applicable.

Funding

The present study was funded by the General Directorate of Academic Personnel Affairs of the National Autonomous University of Mexico (grant no. PAPIIT-202422) and Fundacion UNAM (grant no. UNA 290722 7Y5).

Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Authors' contributions

GGV wrote the manuscript and performed the western blotting, colony formation and migration assays. MRM performed the viability assay, western blotting and IC_{50} calculation. GGV and MRM confirm the authenticity of all the raw data. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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