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SHORT COMMUNICATION

Cytotoxicity of Salvigenin from *Asterohyptis stellulata* in Combination with Clinical Drugs Against Colorectal Cancer

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Abstract

Flavonoids, abundant polyphenols in various plant-based sources, exhibit diverse health benefits, particularly in cancer prevention and treatment, attributed to their ability to mitigate oxidative stress. Salvigenin, a naturally occurring trimethoxylated flavone from the aerial parts of *Asterohyptis stellulata* Epling, Lamiaceae, has gained attention for its potential synergistic effects with conventional anticancer drugs. The present study describes the evaluation of salvigenin, a non-cytotoxic flavone ($IC_{50} > 50$ µM), in combination assays with clinical drugs in human colon carcinoma cells (HCT-116), which revealed significant differences as compared to single salvigenin treatments. Remarkably, IC_{50} values of 1.8 and 1.5 µM for the combination of salvigenin with sublethal concentrations of podophyllotoxin and colchicine (0.008 µM), respectively, were observed, indicating an enhancement in its cytotoxicity effectiveness. These findings emphasize the potential of salvigenin-based combination therapies as a promising strategy for colorectal cancer treatment, offering improved therapeutic results with reduced clinical drug doses and associated side effects.

Keywords Antioxidants · Antiproliferative potential · Chemotherapeutic drugs · Cytotoxicity · Natural adjuvants · Polyphenols

Introduction

Colorectal cancer, also known as bowel cancer, is the third most common diagnosis making up about 10% of all type of cancers, and second lethal malignancy due to old age with both strong environmental associations to lifestyle and genetic risk factors (Eom et al. 2021). Surgical resection for localized early stages is commonly executed. In addition, standard treatments include chemotherapy with anticancer drugs and target therapy (monoclonal antibodies as well as angiogenesis and protein kinase inhibitors). Recently, adjuvant therapy with antioxidant natural products, such as flavonoids, has proven to increase the chance of cure on high-risk patients with colon cancer (Namdeo et al. 2020).

Flavonoids are secondary metabolites with widespread presence in different herbal medicinal matrices, vegetables, and fruits, as essential nutraceuticals, where they exert protection to plants against UV radiation, microbe infections, and oxidative stress. In addition, comprehensive research has showed the valuable roles of flavonoids in human health, including anticancer, antihypertensive, or antithrombotic effects. Especially, their anticancer potential has been documented and commonly attributed to their ability to regulate oxidative stress as therapeutic agents via suppressing reactive oxygen species (Slika et al. 2022).

In the viewpoint of colorectal cancer treatments, all flavonoids could have effectiveness, as antiproliferative agents, via diverse mechanisms of action, which include carcinogen inactivation, cell cycle arrest by induction and differentiation of apoptosis, inhibition of angiogenesis, acting as chemosensitizers for reversal of multidrug resistance in cancer cells, or reducing the oxidative stress caused by pharmacological drug treatments (Kapoor et al. 2021).

Salvigenin (**1**) or 5-hydroxy-4′,6,7-trimethoxyflavone ($C_{18}H_{16}O_6$) is naturally occurring in various plant families, mainly including Lamiaceae (Ayatollahi et al. 2009) and Asteraceae (Noori et al. 2013; Serino et al. 2021). This flavone has demonstrated effective cytotoxicity through

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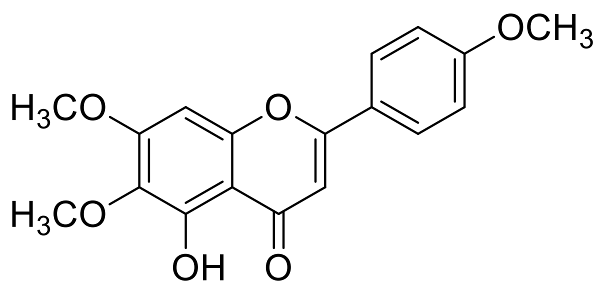
induction of apoptosis in human cancer cell, such as colon adenocarcinoma (HT-29), breast adenocarcinoma (MCF-7), glioblastoma (SF-268), and human kidney epithelial cells (Sarvestani and Sepehri 2016). Salvigenin reduces tumor cell growth *in vivo* and enhanced cellular immune responses (Noori et al. 2013). Recently, it has been shown that co-administration of **1** with doxorubicin induced apoptotic effects via mitochondrial disfunction in colorectal HT-29 and SW948 cancer cell lines (Sarvestani et al. 2018). Therefore, the present investigation evaluated the combinatory effect of salvigenin from *Asterohyptis stellulata* Epling, Lamiaceae, with the potential to induce apoptosis and cell cycle arrest and synergize the activity of therapeutical antitumor drugs to offer an improvement for the treatment of human colon carcinoma cells (HCT-116) by drug dose reduction and the concomitant decreasing of side effects (Araújo et al. 2011; Patel 2021).



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Material and Methods

Aerial parts of *Asterohyptis stellulata* Epling, Lamiaceae (Fig. S1) were collected in the locality of Otates, Municipality of Actopan, State of Veracruz, Mexico (Lat, 19.532963 N; Long, − 96.717062 W; Alt, 488 masl) on January 14, 2023, by A.C. Hernández-Rojas and M. Kilian. Samples of the species were identified by Dr. Hernández-Rojas and deposited at the Herbarium XAL with duplicates (accession number 152242) of the Instituto de Ecology A.C. (INECOL, Xalapa, Veracruz). The plant material (1.4 kg) was extracted by maceration 4 × 24 h each at room temperature with petroleum ether. A yellowish-white solid precipitated (250 mg) from the petroleum ether solution (Fig. S3). This solid was washed with MeOH (3 ×) to remove polar impurities and pigments, as the solid was insoluble in this protic solvent. Subsequently, the clean solid was subjected to recrystallization. Initially, it was dissolved in CH_2Cl_2 (3.5 ml) and, to facilitate a controlled crystallization, a few drops of petroleum ether (0.5 ml) were added. The solution was filtered and allowed to cool at room temperature, resulting in the formation of pale-yellow crystals (125 mg). Further recrystallization (50 mg) with CH_2Cl_2-petroleum ether (9:1) yielded 40 mg of pure salvigenin (**1**). This compound was identified by comparison of its physical constants (Moradkhani et al. 2012) and spectroscopic properties, such as ^{1}H and ^{13}C NMR (Fig. S4), as well as HRMS (Fig. S5), with published values (Ayatollahi et al. 2009).

Salvigenin (**1**): pale yellow crystals, mp 185–187°; ^{1}H NMR (600 MHz, $CDCl_3$) δ 12.77 (s, 1H, C_5-OH), 7.82 (d, $J = 9.0$ Hz, 2H, H-2′ and H-6′), 7.00 (d, $J = 9.0$ Hz, 2H, H-3′ and H-5′), 6.56 (s, 1H, H-3), 6.53 (s, 1H, H-8), 3.96 (s, 3H, C-7, -OMe), 3.92 (s, 3H, C-6, -OMe), 3.88 (s, 3H, C-4′, -OMe); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 182.8 (C-4), 164.1 (C-2), 162.7 (C-4′), 158.8 (C-7), 153.3 (C-4a), 153.2 (C-8), 132.8 (C-6), 128.1 (C-3′, C-5′), 123.7 (C-1′), 114.6 (C-2′,C- 6′), 106.3 (C-5), 104.2 (C-3), 90.7 (C-8a), 61.0 (C-7, -OMe), 56.4 (C-6, -OMe), 55.7 (C-4′, -OMe). HR APCI-TOF–MS *m/z* 329.1016 $[M + H]^+$ (calcd. for $C_{18}H_{17}O_6$ *m/z* 329.1019, δ = − 0.9 ppm), 314.0778 $[M + H - CH_3]^+$, 296.0664 $[314 - H_2O]^+$, 268.0723 $[296 - CO]^+$.

Cytotoxicity and drug combination assays were conducted with salvigenin (**1**) and using human colon carcinoma cells (HCT-116). The cells were cultured in fetal bovine serum medium, 100 U/ml penicillin G, and 100 µg/ml streptomycin at 37 °C under 5% CO_2 atmosphere and 100% relative humidity. Cells were used when they reached 60–70% confluence and were maintained in the logarithmic growth phase. A suspension of 10^4 cells was used. From this suspension, 190 µl was seeded in 96-well plates and 10 µl of different concentrations of test samples in DMSO (10%) was added and the experiments were performed in triplicate. The microplates were incubated for 72 h, and the sulforhodamine B (SRB) method was used. Cell density was determined using an ELISA plate reader at 564 nm. For the combination assays, cells were exposed to test compounds for 72 h. The clinical drugs vinblastine (0.003 µM), podophyllotoxin (0.008 µM), colchicine (0.008 µM), ellipticine (1.6 µM), and doxorubicin (0.7 µM) were individually tested at sublethal concentrations in combination with salvigenin (**1**), which was evaluated at final concentrations of 50, 30, 20, 10, 3, and 1 µM, followed by SRB method. The growth percentage was plotted along with their respective concentrations using Prisma v. 8.01 to obtain the IC_{50} (Moreno-Velasco et al. 2024).

Results and Discussion

The name for the genus *Asterohpytis* was derived from the Greek *Aster* (star) due to the calyx found at the base of the flowers with a stellar-like lobes, a character used to distinguish the genus which, first proposed by Epling, was segregated from the large genus *Hyptis*. It belongs to the subtribe

Hyptidinae that encompasses approximately 19 genera and around 400 species distributed mainly in tropical America (Pastore et al. 2021). *Asterohyptis* is also distinguished from *Hyptis* by its numerous small flowers (reduced white corollas) which are arranged in axillary elongate clusters or spike-like inflorescences, composed of few-flowered verticillasters, in axils of reduced bracts, corolla lobes not thickened, and non-explosive anthers (Figs. S1 and S2). The calyx lobes are subulate or filamentous, often rigid, spreading, corollas weakly 2-lipped with 5 subequal lobes, with the thickened hinge at base of anterior corolla lip (Turner 2011).

Asterohyptis stellulata is a shrub that grows primarily in the seasonally dry tropical biome, frequently in open habitats (Turner 2011). Biogeographical data indicates that its native range is mainly in Mexico, as an endemic species, but its presence is also reported in Central America and Brazil (accessions: IPNI 2024 and GBIF 2024, respectively). In Mexico, its presence in the Pacific slope is remarkable while in the Gulf of Mexico is rare, occurring only in the state of Veracruz (Turner 2011) where populations are not frequently observed, not only because of its restricted natural distribution, but also because of the intensive agricultural and livestock activities in the region. The population studied here consists of few individuals (woody, 5 m high, no recruitment observed), and collected on an abandoned property for almost 15 years (Fig. S1A).

From the ethnobotanical perspective, there are few reports of *A. stellulata*, mainly as a healing plant. Maximino Martínez (1989), on his impressive book—first published in 1939—about the medicinal plants of Mexico, reported the common names and usages known so far, registered as “Cordón de San Antonio,” “hierba del becerro” o “barretero” in the state of Guerrero, and “hierba del ahito” o “té maravilloso” (wondrous tea) in Michoacán. Decoctions of the leaves are used for wound healing; thus, the crude drug is known as “hierba del golpe” (herb to heal bruises) in Morelos (Monroy-Ortíz et al. 2013) where it is also used against indigestion and stomach spasms. The activity of *A. stellulata* was evaluated by Álvarez-Santos et al. (2022) finding antibacterial and antioxidant activity of its phenolic content and promoting closure speed of wounds confirming the traditional use of *A. stellulata* for wound healing. No common name or usage was recorded in the collection locality for *A. stellulata* even though other members of the subtribe Hyptidinae have been used there traditionally for generations, *e.g.*, “hierba del burro”; *Mesosphaerum suaveolens* (L.) Kuntze is commonly used macerated in alcohol mainly to treat gastrointestinal disorders, frequently cultivated in local gardens.

Combinatory antiprolifetarive and palliative effects have been reported for flavonoids on clinic symptoms associated with all therapeutical antitumor drugs, such as nausea, vomiting, diarrhea, mucositis, kidney problems, and peripherial neuropathic pain (Uebel et al. 2019; Fernández et al. 2021). In addition, the protective effect of flavonoids in radiotherapy has also been demonstrated (Wang et al. 2020; Wu et al. 2023). Therefore, these active redox antioxidant polyphenols could provide a double effect in drug combination or co-administration of active agents, potentiating the antitumor effect of clinical chemotherapeutic agents and radiotherapy by controlling oxidative stress and, concurrently, preventing important side effects due to their anti-inflammatory potential and for maintaining the genomic stability (prevention of DNA damage) of normal fast-growing cells, like those in the skin and digestive tract of cancer patients (Slika et al. 2022).

Consequently, for drug combination assays with salvigenin (**1**), the cytotoxicity of five clinical antitumor drugs in human colon carcinoma cells HCT-116 was initially evaluated (Fig. 1). Table 1 summarizes the half maximal inhibitory concentration (IC_{50}) values for the tested clinical drugs in this cell line as well as the cell viability in their combination assays with salvigenin by using the sulforhodamine B colorimetric assay (Moreno-Velasco et al. 2024). Vinblastine had a IC_{50} of 0.23 μM, podophyllotoxin 0.97 μM, colchicine 0.2 μM, ellipticine 9 μM, and doxorubicin 3.2 μM. Salvigenin

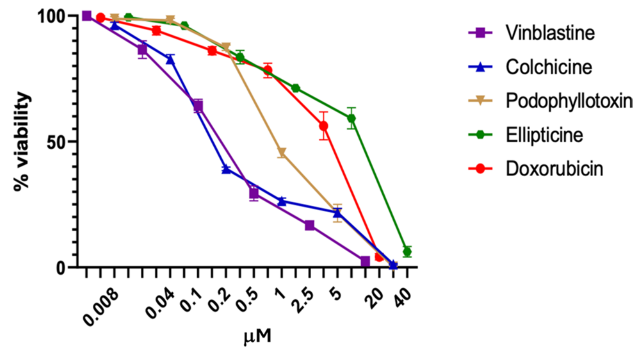

Fig. 1 Cell viability assays in human colon carcinoma cells (HCT-116 line) with clinical antitumoral drugs

Table 1 Cytotoxicity (IC_{50}) for clinical drugs and combination assays with salvigenin (**1**) in human colon carcinoma cells (HCT-116 line) by using the sulforhodamine B colorimetric assay

	IC_{50} (μM)	
	Drug	Salvigenin + drug
Colchicine	0.20 ± 0.02	1.5 ± 0.1
Podophyllotoxin	0.97 ± 0.06	1.8 ± 0.2
Vinblastine	0.23 ± 0.02	4.7 ± 1.3
Ellipticine	9.00 ± 0.05	5.9 ± 0.7
Doxorubicine	3.20 ± 0.13	17.3 ± 1.7

IC_{50} for salvigenin alone: > 50 μM; Combination assay: vinblastine 0.003 μM, colchicine or podophyllotoxin 0.008 μM, ellipticine 1.6 μM, doxorubicin 0.7 μM, and 1–50 μM salvigenin for IC_{50} calculations (tested compound **1** + antitumor drug) showed no effects as a cytotoxic agent with a $IC_{50} > 50$ µM (Fig. 2), in agreement with previous results where flavonoids displayed low cytotoxicity in the HCT-116 line (Fernández et al. 2021). This is an important requirement for the execution of the proposed combination assays with clinical drugs to identify any potentiation effect of salvigenin with the combined antitumor drugs in this HCT-116 line (Moreno-Velasco et al. 2024). Therefore, the potentiation of the cytotoxicity with the individual isolated compound **1** at the concentrations of 50, 30, 20, 10, 5, 3, and 1 µM was investigated with sublethal doses of the tested clinical drugs (0.003 µM for vinblastine, 0.008 µM for colchicine and podophyllotoxin, 1.6 µM for ellipticine, and 0.7 µM for doxorubicin).

A dose–response curve was obtained and the IC_{50} value for the combination of salvigenin and the five tested antitumor drugs was calculated (Fig. 2). The combination assays in HCT-116 cell line exhibited statistically important differences with all tested drugs with respect to single treatment of salvigenin (Fig. 2). The number of surviving cells was compared with an untreated control of salvigenin after 48 h. A significant enhancement for salvigenin cytotoxicity even at 3 µM was observed with all drugs. For instance, while podophyllotoxin at 0.003 µM produced no cell death, supplementation with 3 and 30 µM of salvigenin induced an inhibition of cell viability to 10% and 20%, respectively (Fig. S6). The best IC_{50} values for salvigenin with the tested samples were 1.8 µM for the combination with podophyllotoxin and 1.5 µM for the combination of with colchicine, in contrast to the IC_{50} value of 4.7 µM for the combination with vinblastine, 5.9 µM for the combination with ellipticine, and 17.3 µM for the combination with doxorubicin. The combinations of a subinhibitory dose of podophyllotoxin and colchicine increased the cell death about 30-folds as compared to salvigenin alone. Similar results were previously described for the combination of apigenin (IC_{50} 0.98 µM) and luteolin (IC_{50} 0.99 µM) in combination with the clinical drug 5-fluorouracil in HTC-116 colon carcinoma cells (Fernández et al. 2021). The antiproliferative effect of salvigenin-induced cell death in combination assays, due to reactive oxygen species scavenging, could be associated with the potentiation of apoptotic pathways as previously described for flavonoids (Namdeo et al. 2020; Kapoor et al. 2021; Sarvestani et al. 2018; Patel 2021).

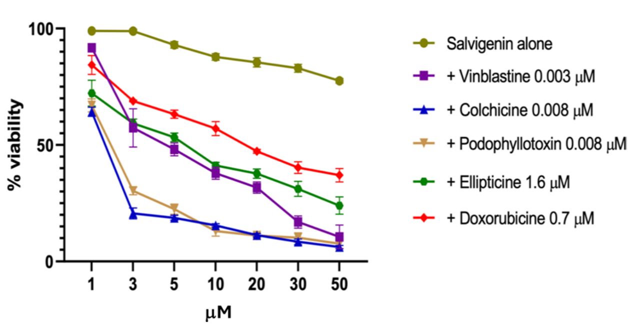

Fig. 2 Combinatorial assays with subinhibitory concentrations of clinical antitumoral drugs with salvigenin (**1**) at various concentrations in human colon carcinoma cells (HCT-116 line)

In conclusion, a combination of salvigenin (**1**) with an antitumoral drug induces antiproliferation activity and enhances cell death on colorectal cancer cells which might allow a reduction of malignant side effects by dose lowering of therapeutic drugs.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s43450-024-00549-0.

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Author Contributions BARC: isolation, chemical analysis, cytotoxicity assays, and writing of the first draft; ACHR: collection of plant material, botanical identification, preparation of dried herbarium specimens, and field ethnobotanical observations and notes on the plant material; MFS: cytotoxicity assays; RPM and MFS: conceptualization of the project and technical supervision. All authors have read the final manuscript and approved its submission. This article was taken from the M. Sc. thesis of Briand André Rojas-Castaño, Programa de Maestría y Doctorado en Ciencias Químicas, UNAM.

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Data Availability Data and material will be made available on request.

Declarations

Ethics Approval Not applicable.

Competing Interests The authors declare no competing interests.

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