Pharmaceutical Sciences and Drug Design
ISSN: 3062-4428
2026, Volume 6, Issue 1, Page No: 134-151

Available online at: www.galaxypub.co/page/journals

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Chemodiversified Brazilin Derivatives Suppress Migration and FAK Signaling in Breast Cancer Cells

Wei Chen[1]*, Li Zhang[1]

[1]Department of Drug Design and Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Peking University, Beijing, China.

***E-mail** ✉ wei.chen@outlook.com

Received: 10 December 2025; **Revised:** 16 March 2026; **Accepted:** 18 March 2026

ABSTRACT

Breast cancer is the most frequently diagnosed malignancy in women and remains the leading cause of cancer-related mortality worldwide. The treatment of invasive forms of this disease is particularly problematic due to the toxicity and side effects associated with conventional chemotherapeutic agents. Natural products have therefore gained attention as promising candidates for anticancer drug development; among them, the homoisoflavonoid brazilin has been reported to exhibit several biological activities, including anti-tumor and anti-inflammatory effects. In this work, brazilin was isolated from the heartwood of *Haematoxylum brasiletto*. Subsequently, structural modification was achieved by semi-synthesis, introducing three methyl or acetyl groups onto the parent brazilin scaffold. The chemical identities of brazilin and its derivatives were confirmed by spectroscopic analyses (^{1}H NMR and ^{13}C NMR), and their purities were determined by optical rotation measurements. The biological activity of brazilin and its derivatives was then investigated in three mammary-derived cell lines: the triple-negative breast cancer (TNBC) line MDA-MB-231, the estrogen receptor-positive ERα(+) MCF7 line, and the non-tumorigenic MCF10A epithelial cell line. Cell viability was assessed using MTT assays, migratory behavior was analyzed through wound-healing assays, and focal adhesion kinase (FAK) activation was examined by Western blotting. The MTT results demonstrated that both MCF7 and MDA-MB-231 cancer cells were sensitive to the compounds at 20 µM, whereas no cytotoxicity was observed in MCF10A cells. The strongest response was observed in MDA-MB-231 cells, where brazilin exhibited an IC50 value of 49.92 µM, while in MCF7 cells, the brazilin-(OAc)3 derivative showed an IC50 of 49.97 µM. These effects were dependent on both dose and exposure time and were accompanied by reduced cell migration and suppression of FAK activation.

Keywords: FAK activation, Cell migration, Chemodiversification, Semi-synthesis, Brazilin

How to Cite This Article: Chen W, Zhang L. Chemodiversified Brazilin Derivatives Suppress Migration and FAK Signaling in Breast Cancer Cells. Pharm Sci Drug Des. 2026;6(1):134-51. https://doi.org/10.51847/KgeVIGJpDV

Introduction

Natural products represent a fundamental and enduring resource in drug discovery and pharmaceutical development [1, 2]. According to Newman and co-workers, approximately 75 compounds (41%) among anticancer drugs approved between 1946 and 2019 originated from or were inspired by natural sources [3]. These sources include a wide range of organisms such as bacteria, algae, and plants, and yield diverse classes of secondary metabolites, including tannins, stilbenes, lignans, phenolic acids, and flavonoids. Among these, flavonoids are particularly abundant and display strong biological activity [4]. Numerous studies have documented the anticancer properties of flavonoids, highlighting their ability to modulate reactive oxygen species (ROS), induce cell cycle arrest, promote programmed cell death, and inhibit the proliferation and metastatic potential of tumor cells [5, 6]. In addition, several isoflavonoids, such as genistein, daidzein, biochanin A, and glycitein, are advancing into clinical evaluation for the development of anti-invasive cancer therapies [7].

Brazilin [(6aS, 11bR) -7, 11b-dihydro-6H-indeno [2,1-c] chromene-3, 6a, 9, 10-tetrol] is a naturally occurring tetracyclic homoisoflavonoid characterized by a chroman core fused in a cis configuration with a 2,3-dihydro-1H

indene unit [8]. This compound is predominantly found in the heartwood of *Caesalpinia sappan* L. (Leguminosae), native to Southeast Asia, and has also been isolated from ethanolic extracts of *Haematoxylum brasiletto* (Fabaceae) heartwood [9]. *H. brasiletto*, commonly known as "Palo de Brasil", is traditionally used in the preparation of infusions, beverages, and medicinal extracts for the treatment of chronic ailments [8]. Extracts containing brazilin from this species have shown cytotoxic activity against several cancer cell lines, including A549, LS180, HeLa, SiHa, MDA-MB-231, and NCI-H1299, with a general reduction in cell viability across all tested models [9]. Previous findings further indicate that brazilin can interfere with epithelial–mesenchymal transition (EMT) processes by downregulating markers such as vimentin and Twist, thereby reducing invasive behavior in breast cancer cells [10]. Despite these promising results, the role of brazilin in breast cancer progression and metastasis is still not fully understood and remains an active area of investigation.

Breast cancer is the most frequently diagnosed cancer type and the primary cause of cancer-related deaths in women globally. In 2020, women aged between 45 and 55 years accounted for more than two million new cases, with mortality exceeding half a million [11]. Among the different molecular subtypes, triple-negative breast cancer (TNBC) is considered the most aggressive form, often characterized by rapid disease progression, metastasis, and resistance to conventional chemotherapy [12]. Metastasis, one of the defining hallmarks of cancer [13], is responsible for the majority of cancer-related deaths [14]. This multistep biological process involves detachment of tumor cells through loss of cell–cell and cell–extracellular matrix (ECM) adhesion, acquisition of migratory and invasive properties, penetration into surrounding tissues, entry into the bloodstream, and eventual colonization of distant organs through extravasation, where secondary tumors are formed [15].

Cell migration is an early and essential event in metastasis and relies on extensive cytoskeletal remodeling, particularly the polarization of actin filaments and formation of membrane protrusions that drive movement [16]. Moreover, adhesion structures such as focal adhesions (FAs) provide both structural guidance and mechanical stability during migration. The regulation of actin dynamics and focal adhesion turnover is mediated by signaling cascades involving kinases such as focal adhesion kinase (FAK) and Src [17]. Although clinical strategies such as surgery, radiotherapy, hormonal therapy, and targeted treatments are available, chemotherapy remains the main therapeutic approach for TNBC [18]. Nevertheless, nearly 90% of patients receiving chemotherapy experience significant adverse effects [19, 20]. This highlights an urgent need for the development of safer and more effective therapeutic agents for invasive breast cancer.

Previous reports indicate that brazilin can block migration of vascular smooth muscle cells (VSMCs) activated by platelet-derived growth factor (PDGF), with the suggested mechanism involving inhibition of the PDGF receptor (PDGFR)–Src–ERK1/2–Akt signaling pathway [21]. Another study showed that when brazilin was combined with doxorubicin (DOX), it reduced migration in HER2-overexpressing MCF7 breast cancer cells (MCF7/HER2), potentially through suppression of proteins such as MMP-2, MMP-9, HER2, Rac1, and p120 [22]. In addition, recent work examined brazilin's influence on breast cancer invasiveness in both in vitro and in vivo settings. The findings revealed that brazilin reduced proliferation, migratory behavior, and invasive capacity in 4T1 and MDA-MB-231 cell lines; in animal models, treatment led to improved survival, fewer metastatic deposits, and a smaller tumor burden [23].

Despite the importance of natural compounds as a source of anticancer agents, many exhibit unfavorable drug-like properties, including poor solubility, limited systemic absorption, low bioavailability, and extensive metabolic instability, all of which hinder successful clinical development. For this reason, structural modification is regarded as a critical step in optimizing drug candidates [24]. Semi-synthesis is commonly employed to enhance biological performance and target specificity by chemically modifying the parent scaffold, typically via addition, substitution, or removal of functional groups [25]. Such structural tuning can significantly alter both physicochemical behavior and pharmacological activity, often improving therapeutic efficacy, as demonstrated by cabazitaxel (Jevtana®, Sanofi, Hong Kong), a methoxylated derivative of docetaxel modified at C-7 and C-10 [26]. Likewise, halichondrin B, originally isolated from *Halichondria okadai* and investigated for breast cancer treatment, was later structurally simplified to generate eribulin mesylate, which exhibits enhanced therapeutic activity with reduced toxicity [27, 28].

In this work, we aimed to investigate the anti-migratory properties of brazilin and two semi-synthetically modified derivatives in three mammary-derived cell models. Brazilin was first extracted from the heartwood of *H. brasiletto*, followed by chemical diversification through semi-synthesis, yielding brazilin-(OMe)3 and brazilin-(OAc)3. The biological effects of the parent compound and its methylated and acetylated derivatives were assessed in triple-negative breast cancer (TNBC) MDA-MB-231 cells, ERα-positive MCF7 cells, and non-

cancerous MCF10A mammary epithelial cells. We further evaluated cell viability, migratory capacity, and focal adhesion kinase (FAK) signaling activity. Overall, the tested compounds showed cell line–dependent effects, with the strongest response observed in MDA-MB-231 breast cancer cells.

Materials and Methods

Chemistry general information

All chemicals and solvents used throughout the study were of analytical-grade quality and were used directly as supplied unless otherwise indicated. Reaction monitoring during synthesis was performed using Merck silica gel 60 F254 aluminum TLC plates (Merck, KGaA, Darmstadt, Germany). Flash chromatography purification was carried out using Silica Flash 60 (230–400 mesh; Merck, KGaA, Darmstadt, Germany), with compound visualization achieved using iodine vapor exposure or potassium permanganate ($KMnO_4$) aqueous staining.

NMR spectra for hydrogen and carbon (^{1}H-NMR and ^{13}C-NMR) were recorded on a Bruker 500 MHz instrument using CD3OD or CDCl3 as solvents, with tetramethylsilane (TMS) as the internal standard. Chemical shifts (δ) are expressed in ppm, while coupling constants (J) are reported in Hz. Signal multiplicities follow standard NMR notation. Melting point values were obtained using a MEL-TEMP® 3.0 device and are reported uncorrected. Optical rotation measurements were performed on a Perkin Elmer 341 polarimeter (Perkin Elmer, Waltham, MA, USA) at 589 nm using methanol (MeOH) as solvent. High-resolution fast atom bombardment mass spectrometry in positive ion mode (HRFAB+ MS) was conducted using a JEOL MStation MS-700 instrument (JEOL, Tokyo, Japan) for structural confirmation of the synthesized derivatives.

Compound Brazilin isolation

The heartwood of *H. brasiletto* was harvested in Mochitlan, Guerrero, Mexico, at coordinates 99°21′19.03” W; 17°29′03.27” N and an altitude of 1042 msnm.

Purification of 6aS,11bR-1 or (+)-brazilin:

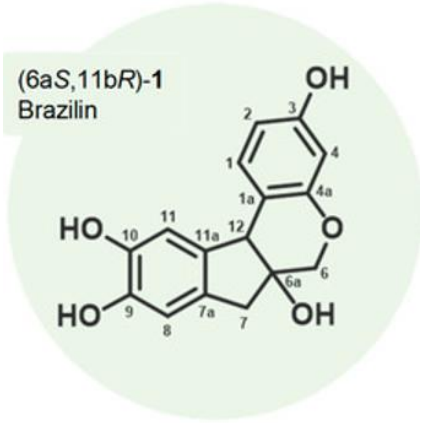
(1) The powdered heartwood of *H. brasiletto* was subjected to maceration in ethanol (EtOH) at ambient temperature for a period of seven days, with intermittent agitation. The resulting ethanolic solution was concentrated under reduced pressure using a rotary evaporator to obtain the crude total extract.

(2) This crude extract was re-dissolved in 250 mL of a hydroalcoholic mixture (H_2O/EtOH, 3:2) and subsequently partitioned through liquid–liquid extraction using n-hexane and dichloromethane (CH_2Cl_2), yielding fractions of low and intermediate polarity, respectively. These fractions were stored in amber vials at −4 °C until further use.

(3) The crude material was subjected to flash column chromatography using a gradient system of CH_2Cl_2/EtOAc/MeOH (9:1:0.5), resulting in 11 primary fractions labeled 3A–3K.

(4) Fraction 3B was further fractionated by flash chromatography using n-hexane:EtOAc:MeOH (45:50:5), producing 8 subfractions designated 4A–4H.

The final purification step for fraction 4C, using EtOAc:CH_2Cl_2:MeOH (70:30:10, supplemented with 50 µL formic acid), yielded compound (5), brazilin. The ^{1}H and ^{13}C NMR data of brazilin, shown in **Figure 1b**, are consistent with previously reported values [29].



a)

Position	^{1}H (J given in Hz)	^{13}C
1	7.18, d (8.4)	132.42
1a		115.74
2	6.47, dd (8.3, 2.5)	110.15
3		155.86
4	6.30, d (2.5)	104.44
4a		157.98
6	3.69, d (11.4) 3.93, dd (11.4, 1.4)	71.02
6a		78.27
7	2.77, d (15.6) 3.02, d (15.6)	43.06
7a		131.53
8	6.71, s	113.06
9		145.79
10		145.48
11	6.60, s	112.62
11a		137.62
12	3.94, s	51.21

b)

Figure 1. Brazilin isolated from *H. brasiletto*: (a) Molecular structure, and (b) spectroscopic characterization (^{1}H NMR and ^{13}C NMR) of brazilin.

Structural elucidation of Brazilin-1
(6aS,11bR)-9,10-dihydroxy-6,6a,7,11b-tetrahydroindeno[2,1-c]chromene-3,6a-diol
(+)-Brazilin ($C_{16}H_{14}O_5$; molecular weight: 286.08) (purity: 91%)
m.p.: 119–121 °C. $[\alpha]^{25D}$ +69.2 (c 1.00, MeOH).
^{1}H-NMR (500 MHz, CD_3OD): δ = 7.18 (d, J = 8.4 Hz, 1H), 6.71 (s, 1H), 6.60 (s, 1H), 6.47 (dd, J = 8.3, 2.5 Hz, 1H), 6.30 (d, J = 2.5 Hz, 1H), 3.96 (s, 1H), 3.93 (dd, J = 11.3, 1.4 Hz, 1H), 3.69 (d, J = 11.3 Hz, 1H), 3.02 (d, J = 15.6 Hz, 1H), and 2.77 (d, J = 15.6 Hz, 1H) ppm.
^{13}C-NMR (125 MHz, CD_3OD): δ = 157.98, 155.86, 145.79, 145.48, 137.62, 132.42, 131.53, 115.74, 113.06, 112.62, 110.15, 104.44, 78.27, 71.02, 51.21, and 43.06 ppm.
HRMS (FAB+): m/z [M + H]+ Calculated for $C_{16}H_{15}O_5$: 287.0919; observed: 287.0991.

Semi-synthesis of Brazilin-(OMe)3 and Brazilin-(OAc)3
3',9',10'-O-trimethylbrazilin (brazilin-(OMe)3)
(6aS,11bR)-3,9,10-trimethoxy-7,11b-dihydroindeno[2,1-c]chromene-6a(6H)-ol
A solution of brazilin (40 mg, 0.14 mmol) in acetone (3.5 mL) at room temperature was treated with potassium carbonate (K_2CO_3) (57.9 mg, 0.4191 mmol) and methyl iodide (MeI) (99.16 mg, 0.6986 mmol). The reaction was stirred at room temperature for 24 h. The resulting crude mixture was purified via column chromatography using an 80:20 EtOAc/hexane system, affording the product [28.8 mg (52%)] as a yellow oil.
^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.28 (d, J = 8.5 Hz, 1H), 6.76 (s, 1H), 6.71 (s, 1H), 6.63 (dd, J = 8.4, 2.6 Hz, 1H), 6.46 (d, J = 2.6 Hz, 1H), 4.10 (s, 1H), 4.01 (dd, J = 11.3, 1.5 Hz, 1H), 3.89 (dd, J = 13.2, 2.0 Hz, 1H), 3.82 (s, 3H), 3.81 (s, 3H), 3.76 (s, 3H), 3.23 (d, J = 15.8 Hz, 1H), 2.86 (d, J = 15.8 Hz, 1H), and 2.52 (br s, 1H) ppm.
^{13}C NMR (125 MHz, $CDCl_3$): δ = 159.63, 154.59, 148.94, 148.65, 136.31, 131.31, 130.77, 114.60, 109.10, 108.66, 107.90, 102.21, 77.72, 70.50, 56.32, 56.28, 55.56, 50.73, and 41.57 ppm.
3',9',10'-O-triacetylbrazilin (brazilin-(OAc)3)
(6aS,11bR)-6a-hydroxy-6,6a,7,11b-tetrahydroindeno[2,1-c]chromene-3,9,10-triyl triacetate
A solution of brazilin (50 mg, 0.17 mmol) in tetrahydrofuran (THF) (2.5 mL) at room temperature was combined with sodium bicarbonate ($NaHCO_3$) (44 mg, 0.5238 mmol) and acetic anhydride (Ac_2O) (89.15 mg, 0.8733 mmol). The reaction mixture was stirred for 6 h at room temperature. The crude product was purified by column chromatography (EtOAc: hexane, 65:50), yielding [24 mg (48%)] of a yellow oil.
^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.34 (d, J = 8.4 Hz, 1H), 7.07 (d, J = 1.0 Hz, 1H), 7.03 (s, 1H), 6.79 (dd, J = 8.3, 2.4 Hz, 1H), 6.68 (d, J = 2.4 Hz, 1H), 4.19 (s, 1H), 4.04 (dd, J = 11.5, 1.6 Hz, 1H), 3.84 (d, J = 11.5 Hz, 1H), 3.30 (d, J = 16.3 Hz, 1H), 2.94 (d, J = 16.3 Hz, 1H), 2.58 (br s, 1H), 2.29 (s, 3H), and 2.26 (s, 6H) ppm.
^{13}C NMR (125 MHz, $CDCl_3$): δ = 169.59, 168.67, 168.57, 154.32, 150.47, 142.34, 141.69, 141.32, 137.60, 131.29, 120.45, 119.59, 119.08, 115.50, 111.02, 77.52, 70.17, 50.64, 41.23, 21.33, 20.86, and 20.81 ppm.

Cell culture and treatments

MCF10A non-tumorigenic mammary epithelial cells, together with ERα-positive MCF7 and triple-negative breast cancer MDA-MB-231 cells (ATCC, Manassas, VA, USA), were maintained in a 1:1 mixture of DMEM/F12 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin G/streptomycin (Gibco, Waltham, MA, USA). Cultures were kept at 37 °C under a humidified atmosphere containing 5% CO_2.

Before exposure to compounds, serum deprivation was applied for 4 h in MCF10A cells and for 24 h in MDA-MB-231 and MCF7 cells, followed by washing with sterile PBS. Treatments were then carried out using brazilin (1), 3',9',10'-O-Trimethylbrazilin (2), or 3',9',10'-O-Triacetylbrazilin (3) at concentrations ranging from 2.5 µM to 40 µM in DMEM/F12 medium for the indicated experimental periods.

Viability assays

Cells were seeded at a density of 10^5 cells per well in 96-well plates and left to adhere overnight. The following day, cells were incubated either with fresh medium alone or with graded concentrations of brazilin, brazilin-$(OMe)_3$, and brazilin-$(OMe)_3$, starting from 40 µM/mL and maintained for 48 h. Staurosporine and DMSO were included as control conditions.

After treatment, 20 µL of MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was added to each well and gently mixed. After a 4 h incubation, the medium was removed and replaced with 100 µL of acidified isopropanol to solubilize the formazan crystals.

Absorbance was recorded at 630 nm using a Thermo Scientific™ Multiskan™ FC microplate reader. Cell viability was calculated by dividing the absorbance of treated samples by that of untreated controls and multiplying by 100 to yield a percentage viability relative to the control.

Cell migration assays

Confluent monolayers of MDA-MB-231, MCF7, and MCF10A cells were established in 60 mm culture dishes containing complete DMEM/F12 medium. Before scratch induction, cells were serum-starved and exposed to 10 µM Cytosine β-D-Arabinofuranoside (AraC) for 2 h to suppress proliferation during the assay.

A linear wound was then created using a sterile 200 µL pipette tip. Detached cells were removed by two washes with ice-cold sterile PBS, after which fresh DMEM/F12 medium was added either without treatment (control/0 condition) or supplemented with 20 µM or 40 µM of the tested compounds.

Wound closure progression was documented using an EVOS FL microscope equipped with a 10× objective. Images were captured immediately after scratching and again 48 h post-treatment; five fields per condition were analyzed. Migration was quantified by measuring wound area using ImageJ 1.44p (NIH, Bethesda, MD, USA).

FAK activation by western blot

After treatment, cells were lysed in ice-cold buffer containing protease and phosphatase inhibitors. Protein lysates (20 ng) were separated using 10% SDS-PAGE and transferred onto nitrocellulose membranes. Non-specific binding sites were blocked using 5% non-fat milk for 2 h at room temperature.

Membranes were incubated overnight at 4 °C with primary antibodies against phosphorylated FAK (44-624G, Invitrogen, Waltham, MA, USA) (1:1000), total FAK (sc-558, Santa Cruz Biotechnology, Dallas, TX, USA) (1:1000), and GAPDH (AC027, ABclonal, Woburn, MA, USA) (1:5000 dilution).

After washing with TBS containing 0.1% Tween-20, membranes were incubated for 2 h at room temperature with HRP-conjugated anti-rabbit secondary antibody (1:5000). Detection was performed using Tanon High-sig ECL Western Blotting substrate (180-5001, ABclonal) and autoradiographic film exposure.

Image acquisition and densitometric analysis were performed using ImageJ 1.54 software (NIH, Bethesda, Rockville, MD, USA).

Statistical analysis

Results are reported as mean ± standard error (SE). Statistical evaluation was performed using one-way ANOVA followed by Dunnett's multiple comparisons test, implemented in GraphPad Prism 9.5.1. A threshold of $P < 0.05$ was considered statistically significant.

Results and Discussion

Brazilin purification from h. brasiletto and semi-synthesis of Brazilin-(OMe)₃ and Brazilin-(OAc)₃

The ground heartwood of *H. brasiletto* served as the starting material for isolating the homoisoflavonoid brazilin, which was obtained in a yield of 0.04% and a purity of 91% **(Table 1).** Identification was confirmed through ^{1}H NMR and ^{13}C NMR spectral analysis **(Figure 1)**.

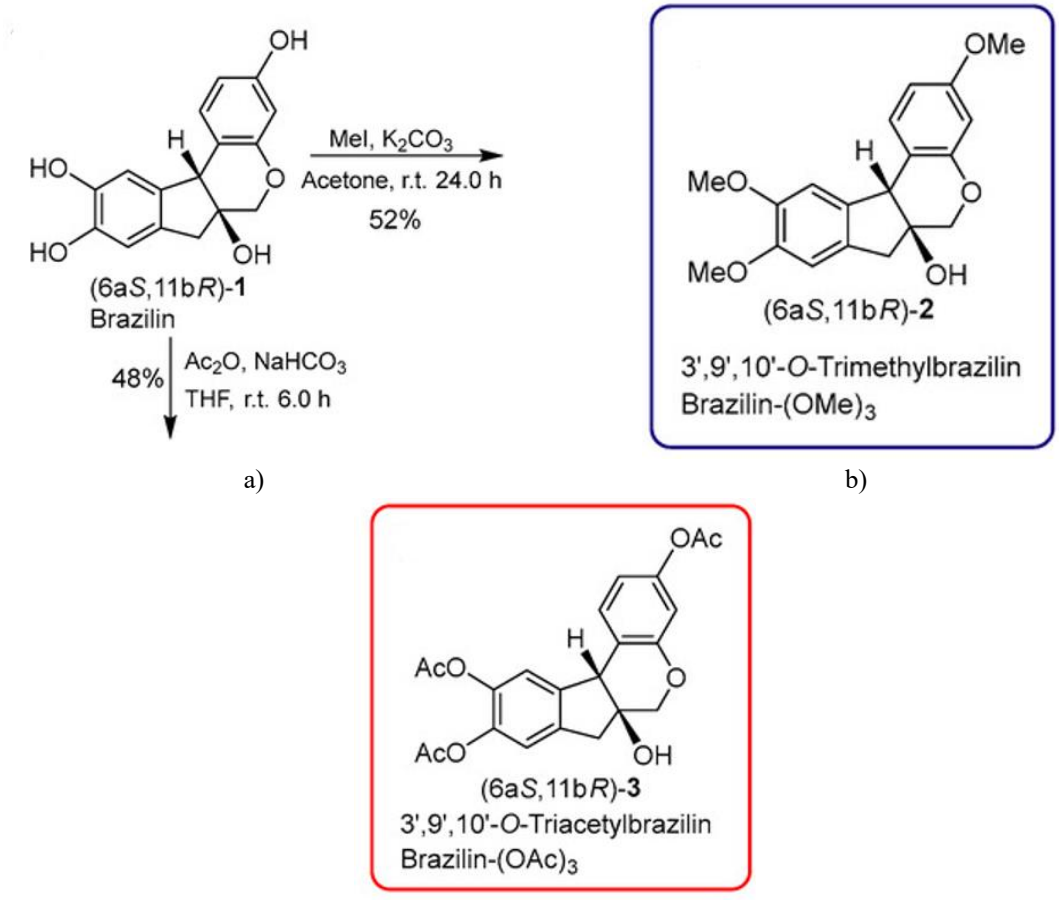
Table 1. Yield and purity of brazilin isolated from *H. brasiletto* heartwood.

Raw Material (g)	Purity (%)	Yield (%)	Yield (g)
1500	91	0.047	0.680

Purified brazilin was subsequently used as a precursor for chemical modification, where three hydroxyl groups at positions 3', 9', and 10' were functionalized to generate two derivatives **(Figure 2).**

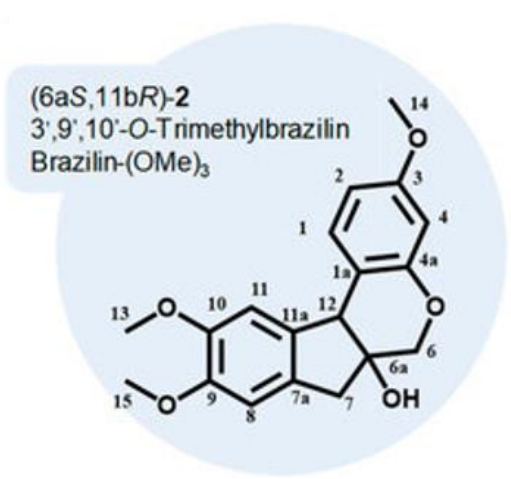
Specifically, treatment of brazilin **(Figure 2a)** with methyl iodide and potassium carbonate in acetone at 25 °C for 24 h yielded brazilin-(OMe)₃ in 52% yield **(Table 2; Figure 2b).** In a separate reaction, brazilin was acetylated with acetic anhydride in the presence of sodium bicarbonate and tetrahydrofuran at 25 °C for 6 h, yielding brazilin-(OAc)₃ with a yield of 48% **(Table 2; Figure 2c).**

Structural confirmation of both derivatives, brazilin-(OMe)₃ and brazilin-(OAc)₃, was achieved using ^{1}H NMR and ^{13}C NMR spectroscopy **(Figure 3)**.

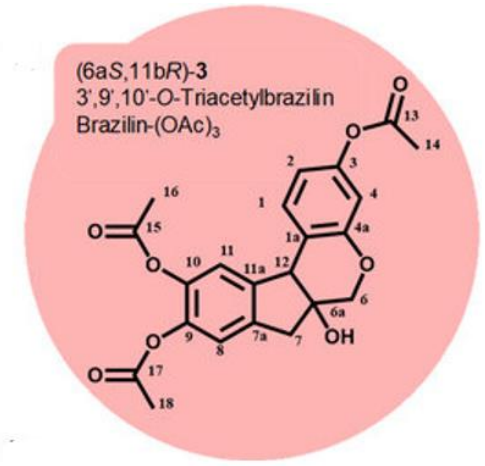


a) b) c)

Figure 2. Synthetic pathway and reaction conditions for chemodiversification of (a) brazilin into (b) brazilin-(OMe)₃ and (c) brazilin-(OAc)₃.



a)



b)

Position	^{1}H (J given in Hz)	^{13}C
1	7.28, d (8.5)	56.28
1a		102.21
2	6.63, dd (8.4, 2.6)	56.32
3		70.5
4	6.76, s	77.72
4a		107.9
6	4.10, s 4.01, dd (11.3, 1.5)	108.66
6a		114.6
7	3.23, d (15.8) 2.86, d (15.8)	130.77
7a		148.65
8	6.46, d (2.6)	148.94
9		155.56
10		159.63
11	6.71, s	131.31
11a		136.31
12	3.89, dd (13.2, 2.0)	109.1
13	3.81, s	55.6
14	3.82, s	50.73
15	3.76, s	41.57

c)

Position	^{1}H (J given in Hz)	^{13}C
1	7.34, d (8.4)	77.52
1a		119.59
2	7.07, d (1.0)	111.02
3		115.5
4	7.03, s	119.08
4a		120.45
6	4.19, s 3.84 d (11.5)	131.29
6a		141.32
7	3.30 d (16.3) 2.94, d (16.3)	141.69
7a		154.32
8	6.79, dd (8.3, 2.4)	168.57
9		168.67
10		169.59
11	6.68, d (2.4)	142.34
11a		150.47
12	4.04, dd (11.5, 1.6)	137.6
13		70.17
14	2.29, s	50.64
15		41.23
16	2.58, s	21.33
17		20.86
18	2.26 s	20.81

d)

Figure 3. Brazilin derivatives. Molecular structures and NMR spectra (^{1}H NMR and ^{13}C NMR) of brazilin-(OMe)$_3$ (a,c) and brazilin-(OAc)$_3$ (b,d).

Table 2. Semi-synthetic yields of brazilin-(OMe)$_3$ and brazilin-(OAc)$_3$.

	Yield (%)	Sample obtained (mg)	Raw material (mg) (Brazilin)
Brazilin-(OMe)$_3$	52	20.8	40
Brazilin-(OAc)$_3$	48	24	50

Brazilin, brazilin-(OMe)3, and brazilin-(OAc)3 effects in cell viability

Cell viability was evaluated after 48 h exposure to brazilin and its two semi-synthetic derivatives, brazilin-(OMe)3 and brazilin-(OAc)3, using two breast cancer cell lines, alongside the non-tumorigenic MCF10A mammary epithelial cells as a reference model. A clear selective reduction in viability was observed in cancer cells, with MDA-MB-231 showing the highest sensitivity, with viability dropping significantly at 20 µM for brazilin and at 80 µM for both derivatives **(Table 3; Figure 4a).**

In MCF7 cells, a significant decrease in viability was observed only at 40 µM for both brazilin and brazilin-(OAc)3 compared with untreated controls **(Table 3; Figure 4b).** By contrast, MCF10A cells showed reduced viability at 20 µM when exposed to brazilin, whereas brazilin-(OMe)3 and brazilin-(OAc)3 affected these non-tumorigenic cells only at concentrations above 40 µM **(Table 3; Figure 4c).**

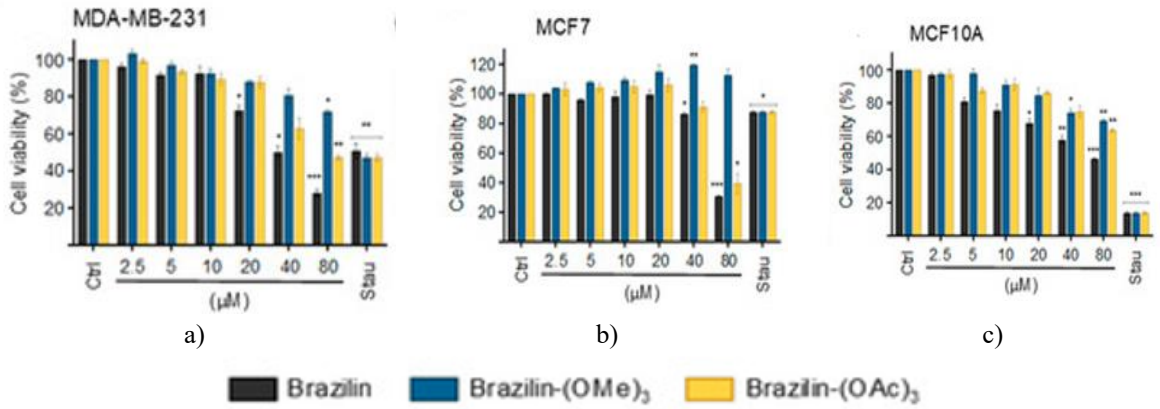


Figure 4. Viability assessment of MDA-MB-231 (a), MCF7 (b), and MCF10A (c) cells following 24 h treatment with brazilin. A one-way ANOVA followed by Dunnett's test was used to compare treatments with the control. Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Staurosporine (Stau) served as a positive control for in vitro apoptosis induction.

Table 3. IC50 values of brazilin and derivatives in breast cancer and non-tumorigenic epithelial cells *.

Cell Line	Brazilin-$(OAc)_3$	Brazilin-$(OMe)_3$	Brazilin
MDA-MB-231	70.75 ± 3.48	> 80 μM	49.92 ± 1.26
MCF7	49.97 ± 3.91	> 80 μM	67.59 ± 0.40
MCF10A	104.09 ± 4.34	> 80 μM	63.64 ± 3.02

Values obtained using non-linear regression analysis.

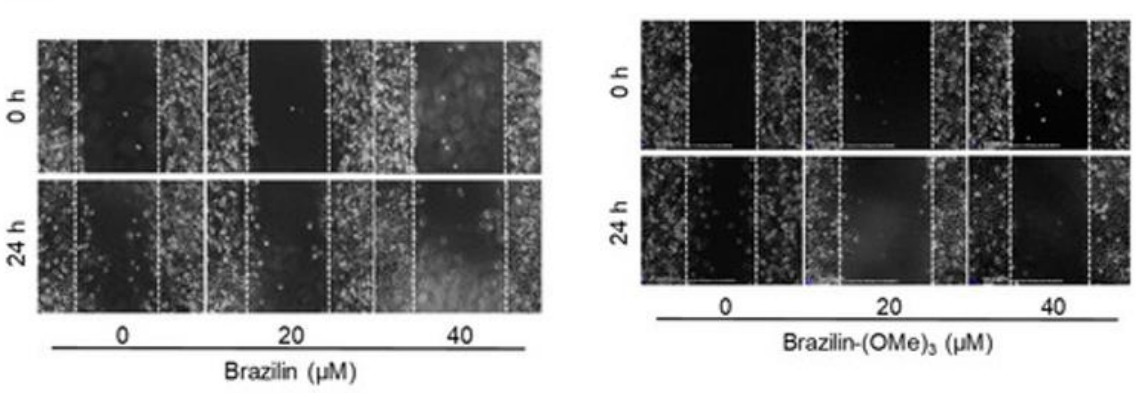
Brazilin, Brazilin-(OMe)3, and Brazilin-(OAc)3 Inhibit breast cancer cell migration

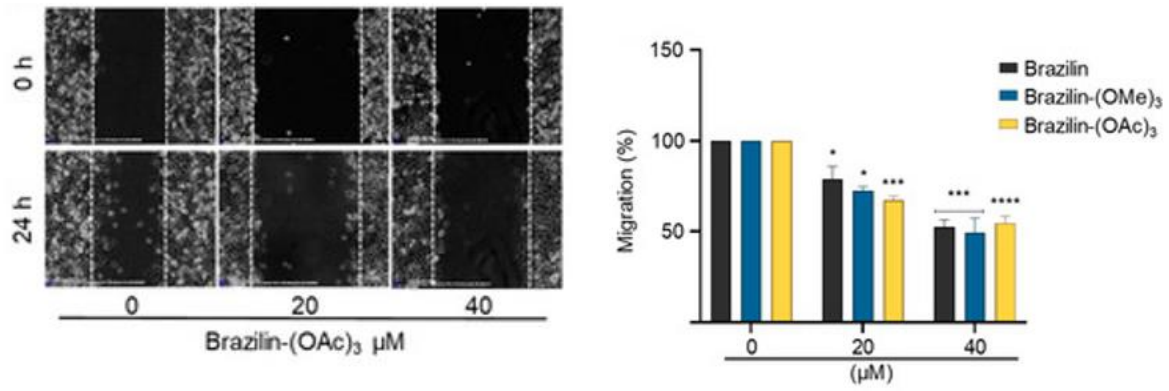
Cell migration is a fundamental step in tumor invasion and metastatic progression and represents a key therapeutic target in oncology [30]. After obtaining the semi-synthetic derivatives of brazilin, their effects on migratory behavior were examined in MDA-MB-231 and MCF7 breast cancer cells, as well as in non-tumorigenic MCF10A epithelial cells.

Migration was quantified using a wound-healing assay by measuring gap closure in cultures treated with 0, 20, or 40 μM of brazilin **(Figure 5a),** brazilin-(OMe)3 **(Figure 5b),** and brazilin-(OAc)3 **(Figure 5c)** over a 24 h period.

KEGG pathway enrichment revealed involvement in a broad range of signaling networks. The most prominent pathways included FoxO, RAS, MAPK, and PI3K-AKT signaling cascades. These were additionally linked to immune and endocrine regulation through pathways such as IL-17 signaling, lectin receptor pathways, relaxin signaling, and hormone-associated pathways, including estrogen and progestin signaling.

Moreover, disease-related pathways were also enriched, including Helicobacter pylori infection, atherosclerosis development, and multiple cancer-associated signaling routes **(Figure 3).**



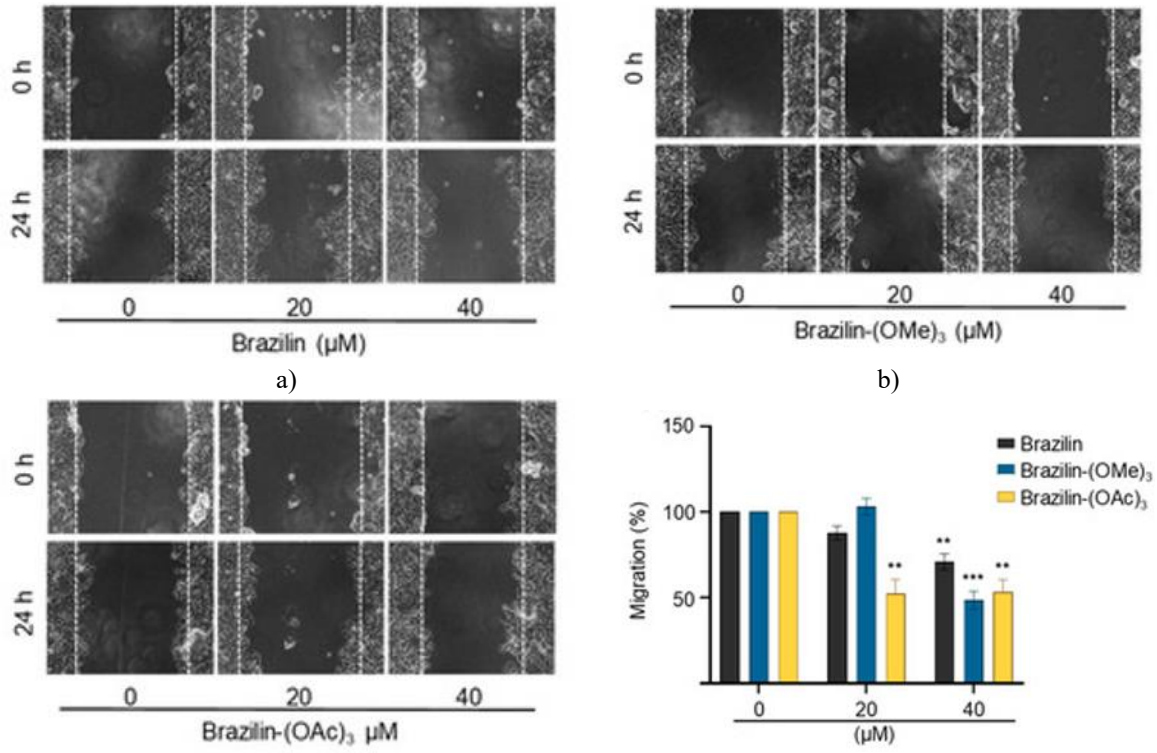


c) d)

Figure 5. Scratch assay evaluating the effect of brazilin and derivatives on MDA-MB-231 cell migration. Cells were first treated with 10 μM Ara-C for 2 h to suppress proliferation, followed by exposure to 20 μM or 40 μM of (a) brazilin, (b) brazilin-(OMe)3, and (c) brazilin-(OAc)3 for 24 h. Representative brightfield images (10× magnification; scale bars = 400 μm). (d) Quantification of migration percentage. Data are from three independent experiments. Statistical analysis used one-way ANOVA with Dunnett's test: * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

In MDA-MB-231 cells, all tested compounds reduced migration in a concentration-dependent manner, with higher doses producing greater inhibition than the untreated condition **(Figure 5d).** At 20 μM, brazilin-(OAc)3 produced the strongest effect, decreasing migration by approximately 30% relative to control **(Figure 5c).** At 40 μM, however, all compounds showed a similar level of inhibition **(Figure 5).**

In MCF7 cells **(Figure 6),** brazilin-(OAc)3 reduced migration by nearly 50% already at 20 μM **(Figure 6c).** In contrast, brazilin-(OMe)3 had no measurable effect at this concentration but became the most effective compound at 40 μM, achieving approximately 50% reduction in migration **(Figure 6b).**



a) b)

c) d)

Figure 6. Wound-healing analysis of brazilin and derivatives on MCF7 cell migration. Cells were pre-treated with 10 μM Ara-C for 2 h and then exposed to 20 μM or 40 μM of (a) brazilin, (b) brazilin-(OMe)3, and (c) brazilin-(OAc)3 for 24 h. Images captured at 10× magnification (scale bars = 400 μm). (d) Migration

quantification. Three independent experiments were analyzed. Statistical significance vs control: ** P < 0.01, *** P < 0.001.

MCF10A cells exhibited lower overall migratory activity than cancer cell lines. Nevertheless, treatment with all three compounds reduced migration by about 50% at 20 µM relative to control, whereas at 40 µM the inhibition decreased to approximately 20% **(Figure 7).**

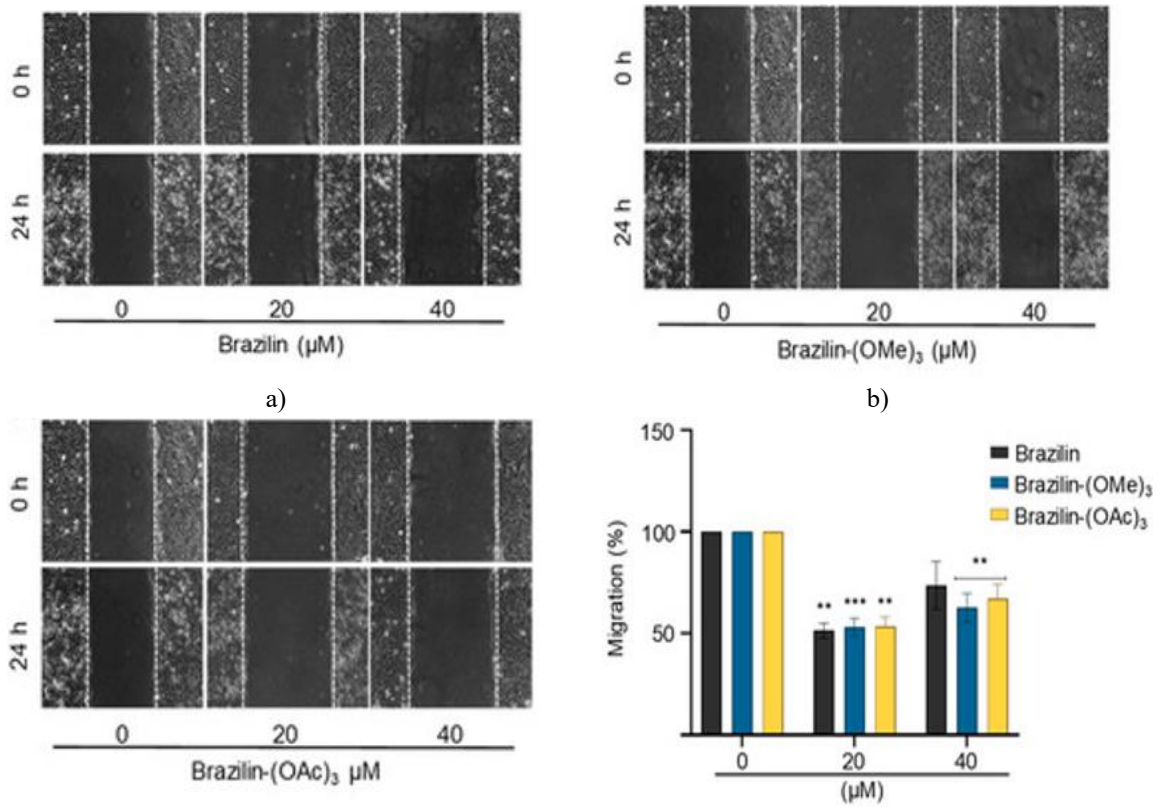


Figure 7. Migration assay of brazilin and derivatives in MCF10A cells. Cells were treated with 20 µM or 40 µM following 2 h Ara-C exposure. Representative images at 10× magnification (scale bars = 400 µm). (d) Quantification of migration percentage. The data represent three independent experiments. Statistical testing used one-way ANOVA with Dunnett's post hoc analysis: ** P < 0.01, *** P < 0.001.

Brazilin, Brazilin-(OMe)3, and Brazilin-(OAc)3 inhibited activation of FAK

Focal adhesion kinase (FAK) functions as a central regulator of cancer cell motility. It is activated within focal adhesion complexes formed through integrin-mediated adhesion and cooperation with multiple intracellular signaling proteins [31]. Its role in migration involves coordinating both the assembly and disassembly of adhesion sites, and activation is primarily driven by autophosphorylation at Y397. Elevated FAK expression has also been associated with enhanced tumor progression and poorer clinical prognosis [32].

To investigate the influence of brazilin and its semi-synthetic derivatives on FAK signaling, Western blot analysis was performed using protein extracts from MDA-MB-231, MCF7, and MCF10A cells exposed for 24 h to concentrations of 2.5, 5, 10, 20, and 40 µM of each compound.

In MDA-MB-231 cells, a non-linear response pattern was observed. At the lowest concentrations (2.5 and 5 µM), both brazilin-(OMe)3 and brazilin-(OAc)3 transiently enhanced FAK activation, whereas exposure from 10 µM onward led to a clear reduction, becoming more evident at 20 µM. Brazilin itself showed minimal effect at lower doses, but noticeable modulation emerged only at 20 and 40 µM **(Figure 8).**

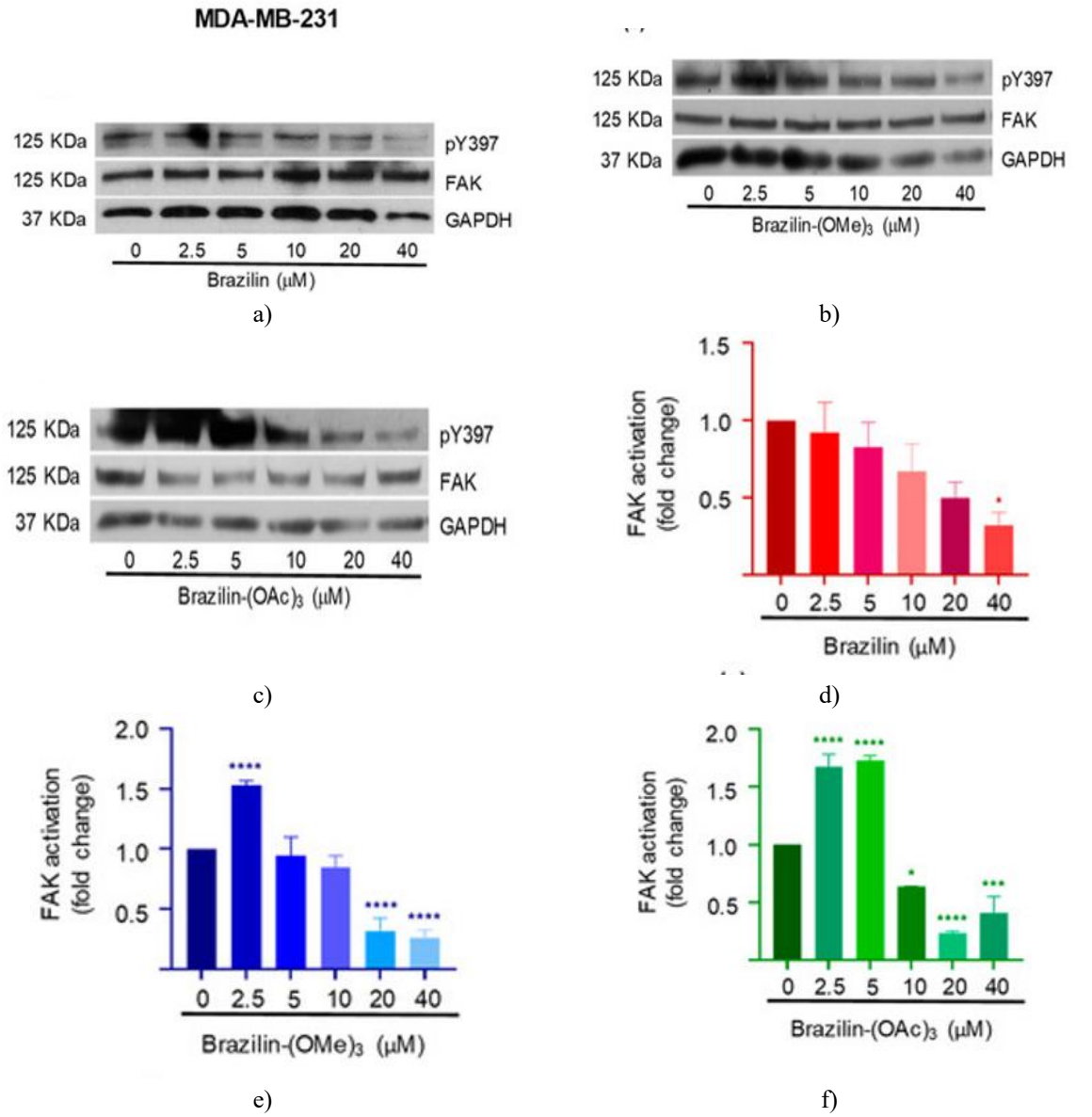


Figure 8. Regulation of FAK activation by brazilin, brazilin-(OMe)3, and brazilin-(OAc)3 in MDA-MB-231 cells. Cells were treated with 2.5–40 μM for 24 h. (a) Western blot analysis for brazilin, (b) brazilin-(OMe)3, (c) brazilin-(OAc)3, and (d–f) Quantitative densitometry and statistical evaluation of protein bands. One-way ANOVA followed by Dunnett's post hoc test was applied. Significance: * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

In MCF7 cells, the response differed depending on the compound and concentration. Brazilin increased FAK activation at lower doses, but at 20 and 40 μM, the phosphorylation levels returned to near baseline control levels **(Figure 9a).** Brazilin-(OMe)3, in contrast, produced a reduction at 2.5 μM, while higher concentrations of both derivatives resulted in elevated FAK activation relative to untreated cells **(Figure 9d).**

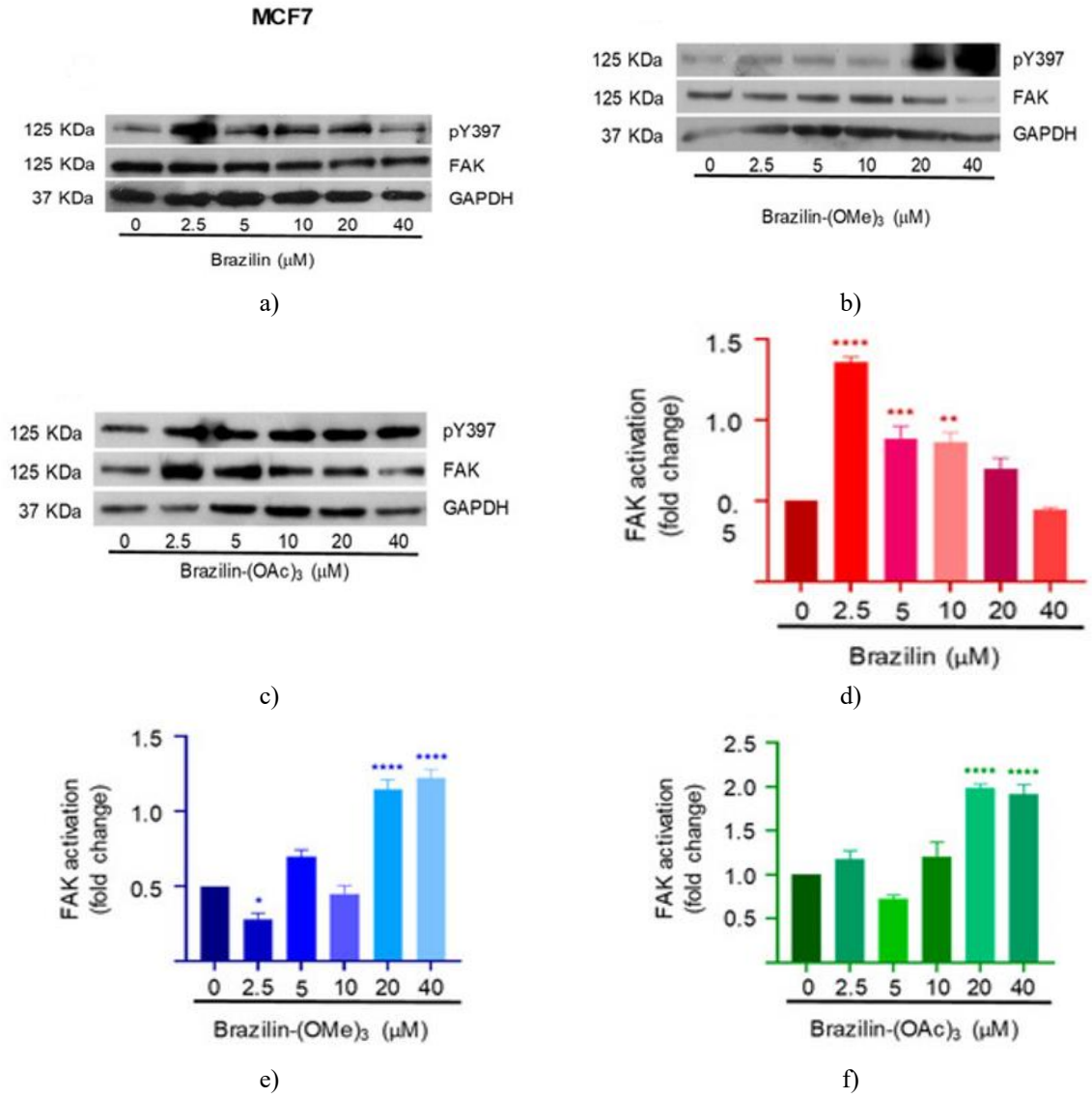
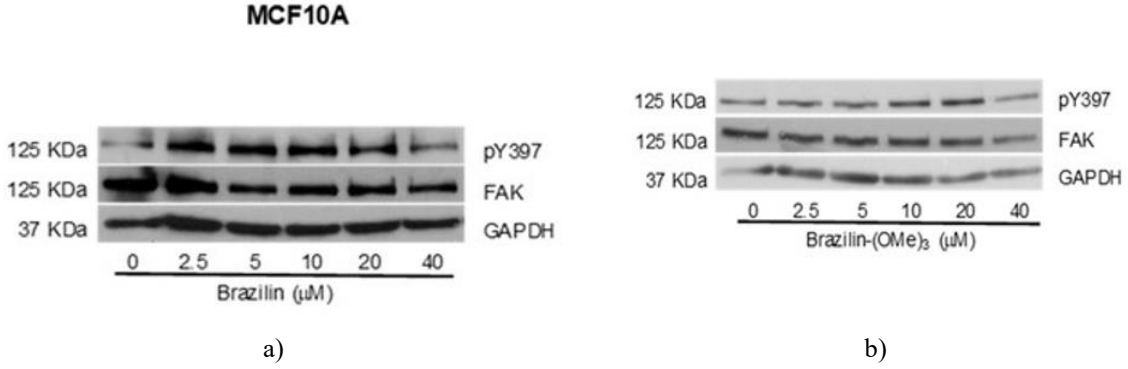


Figure 9. Effects of brazilin, brazilin-(OMe)3, and brazilin-(OAc)3 on FAK activation in MCF7 cells. Cells were exposed to 2.5–40 μM for 24 h. (a) Western blot of brazilin, (b) brazilin-(OMe)3, (c) brazilin-(OAc)3, and (d–f) Densitometric and statistical analyses of bands. Statistical testing used one-way ANOVA with Dunnett's multiple comparison test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

For MCF10A cells, brazilin and brazilin-(OMe)3 both promoted an increase in FAK activation compared with control conditions **(Figures 10a and 10b).** Conversely, brazilin-(OAc)3 produced a progressive, concentration-dependent suppression of FAK signaling **(Figure 10d).**



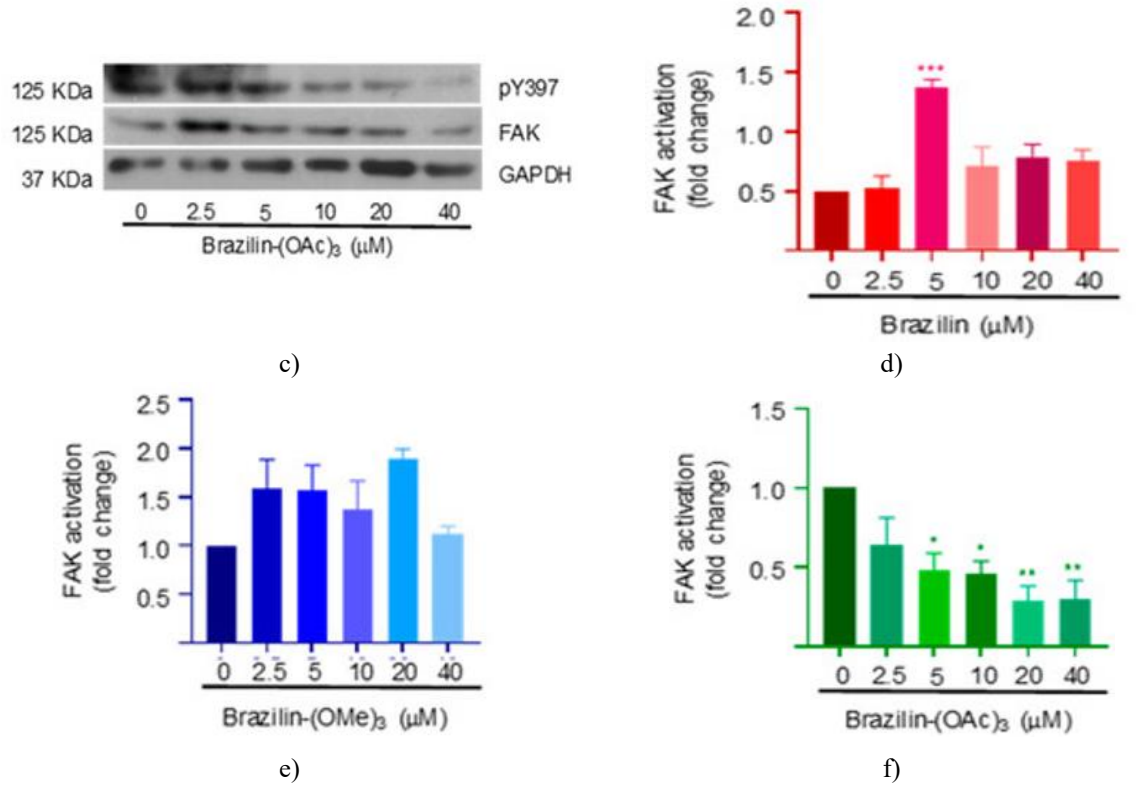


Figure 10. Regulation of FAK activation by brazilin, brazilin-(OMe)3, and brazilin-(OAc)3 in MCF10A cells. Cells were treated with 2.5–40 μM for 24 h. (a) Western blot for brazilin, (b) brazilin-(OMe)3, (c) brazilin-(OAc)3 in MDA-MB-231 cells, and (d–f) Densitometric quantification and statistical evaluation. One-way ANOVA with Dunnett's test was used. Significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Overall, the findings demonstrate that brazilin and its derivatives modulate FAK signaling in a cell-type- and chemical-modification-dependent manner, with the most pronounced and consistent effects observed in the MDA-MB-231 breast cancer model.

Although multiple therapeutic options are currently available, breast cancer remains a leading cause of mortality globally. This persistence underscores the need to continuously identify novel agents with anticancer potential [11, 33]. In this context, interest in phytochemicals has grown considerably due to their notable anti-tumor properties combined with relatively low toxicity profiles, with alkaloids, taxanes, and flavonoids representing the most extensively investigated classes of natural compounds [34].

Brazilin is an isoflavonoid compound originally isolated from *H. brasiletto* [9] and *Caesalpinia sappan* [35]. Previous studies have demonstrated that brazilin exerts broad anticancer activities across multiple tumor models, including suppression of proliferation and cell cycle progression, inhibition of migration, and induction of apoptosis [36, 37]. Nevertheless, its specific role in breast cancer biology has not been thoroughly characterized. Accordingly, the present work examined brazilin obtained from *H. brasiletto* heartwood, together with its chemically modified methoxylated and acetoxylated derivatives, focusing on their effects on migration and FAK signaling in breast cancer models.

Chemical modification strategies are generally employed to enhance compound potency and selectivity, optimize physicochemical, biochemical, and pharmacokinetic characteristics, minimize adverse effects, and reduce the structural complexity of lead molecules. Flavonoids are known to interact with biological systems through mechanisms such as enzyme inhibition and receptor binding [38, 39].

In this study, we first assessed the cytotoxic potential of brazilin and its derivatives using MTT assays in MDA-MB-231 and MCF7 breast cancer cell lines, as well as in non-tumorigenic MCF10A mammary epithelial cells. Distinct IC50 profiles were observed depending on both the compound and the cellular context. Overall, stronger activity was detected in MDA-MB-231 and MCF10A cells, whereas in MCF7 cells, cytotoxicity was mainly associated with brazilin and brazilin-(OAc)3. Notably, the most pronounced response was recorded in MDA-MB-231 cells, where brazilin exhibited an IC50 of 49.92 μM, while brazilin-(OAc)3 reached an IC50 of 49.97 μM in

MCF7 cells **(Figure 4).** These variations likely reflect the distinct molecular and genetic backgrounds of the breast cancer cell lines [40]. MDA-MB-231 cells are characterized by EGFR overexpression, p53 mutation, PTEN loss—which disrupts PI3K/AKT signaling—and activation of NF-κB [41, 42]. In contrast, MCF7 cells are hormone receptor-positive (ER and PR), with proliferation regulated by estrogen and progesterone signaling, and also display overexpression of Bcl-2 and cyclin D, as well as activation of the PI3K/AKT/mTOR pathway [41-43]. Given the broader oncogenic dysregulation in MDA-MB-231 cells, brazilin and its derivatives may act on multiple molecular targets, contributing to the stronger reduction in viability observed in this cell line.

Regarding migration assays, the semi-synthetic derivatives brazilin-(OMe)3 and brazilin-(OAc)3 exhibited more pronounced inhibitory effects compared with the parent compound across all tested conditions **(Figures 5-7).** The observed biological activity may be partially attributed to aromatic structures, which facilitate non-covalent interactions with proteins, including dispersion forces with hydrophobic amino acid residues [44]. In addition, key molecular descriptors such as hydrogen bond donors (HBDs), hydrogen bond acceptors (HBAs), and rotatable bonds strongly influence drug–target interactions. Hydrogen bonding, often mediated by functional groups such as hydroxyl (-OH), stabilizes ligand–protein complexes through specific interactions with active-site residues [45]. Meanwhile, rotatable bonds determine ligand flexibility, where increased conformational adaptability may enhance binding to target sites, although optimization is required for selective interaction with rigid protein pockets [46]. Collectively, these physicochemical properties likely contribute to the differential effects observed on both cell viability and migration in breast cancer models.

Despite the demonstrated inhibitory effects of these compounds on breast cancer cell migration, the underlying molecular mechanisms remain unclear. Therefore, we further investigated their impact on FAK activation in both cancerous and non-tumorigenic epithelial cell lines.

FAK is recognized as a key regulator of cell motility through its role in focal adhesion assembly [47]. Activation of FAK via phosphorylation at Y397 in response to integrin engagement and growth factor signaling triggers downstream pathways associated with cell migration and survival. Importantly, elevated FAK activity has been consistently linked to tumor progression and poor clinical prognosis in cancer patients [48].

A notable outcome of this work was the clear variability in FAK activation across cell types and compound concentrations. In particular, a reduction of approximately 50% in FAK activation was detected only at higher doses of all three compounds in MDA-MB-231 cells, a trend that closely paralleled the observed inhibition of cell migration. This correspondence suggests that, specifically in this triple-negative breast cancer (TNBC) model, brazilin, brazilin-(OMe)3, and brazilin-(OAc)3 may suppress migratory capacity by downregulating FAK signaling. In contrast, at lower concentrations of brazilin-(OMe)3 and brazilin-(OAc)3, an opposite effect was recorded in MDA-MB-231 cells, where FAK activation increased **(Figure 8).**

A similar pattern of enhanced FAK activation was also detected in MCF10A cells across all tested concentrations of brazilin and brazilin-(OMe)3 **(Figure 10).** This behavior may be explained by a hormetic response, in which low doses of cytotoxic agents trigger oxidative stress that paradoxically promotes cellular proliferation and survival signaling in tumor-related contexts [49]. Consistently, increased FAK activity was also observed in MCF7 cells at most concentrations of the three compounds **(Figure 9).**

Specifically, in MCF7 cells, elevated FAK activation at higher doses (20 and 40 μM) of brazilin-(OMe)3 and brazilin-(OAc)3 may be associated with the estrogen receptor status of this cell line. Isoflavonoids share structural similarities with endogenous estrogens, enabling them to interact with estrogen receptors—particularly estrogen receptor beta (ERβ)—and compete with 17β-estradiol at the ligand-binding domain, thereby exerting both agonistic and antagonistic estrogen-like effects [50, 51]. Notably, estrogen signaling through its receptor has been reported to enhance FAK activation in ovarian cancer models [52, 53]. Nevertheless, these observations require further mechanistic validation to clarify this relationship.

Additionally, structural modification of brazilin appears to enhance its pharmacological potential by improving metabolic stability and increasing membrane permeability. Methylation of flavonoids is known to improve stability, as the introduction of methyl groups (-CH3) increases hydrophobicity, thereby facilitating cellular uptake and enhancing bioavailability. This modification also protects hydroxyl groups from metabolic degradation, extends the compound's half-life, and may influence biological activity by enhancing anti-inflammatory and antioxidant effects [54].

Similarly, acetylation, the addition of acetyl groups (-COCH3), can stabilize molecules by reducing the reactivity of functional groups and may improve membrane permeability. However, in certain cases, it can diminish

antioxidant capacity [55]. Together, these chemical modifications represent important strategies for optimizing natural compounds for therapeutic use.

Overall, our findings demonstrate that all three compounds significantly modulate both cell migration and FAK signaling in a dose-dependent manner in MDA-MB-231 cells. This cell line represents triple-negative breast cancer (TNBC), which lacks expression of ER-α/PR and HER2 and is recognized as the most aggressive breast cancer subtype, characterized by early relapse and metastasis to organs such as the lung, liver, and brain, and accounting for more than 50% of breast cancer-related deaths [56, 57].

Given their pronounced activity in this model, these compounds may represent promising candidates for future therapeutic development against breast cancer. Furthermore, advanced delivery strategies such as liposomal or nanoparticle-based formulations have been shown to enhance the bioavailability and tumor-targeting efficiency of natural compounds [58, 59]. Accordingly, future work should include in vivo evaluation of these derivatives to assess their therapeutic efficacy and safety profiles.

However, further studies are still required to fully elucidate the molecular mechanisms through which brazilin and its derivatives influence tumor progression.

Conclusion

The present study demonstrates that brazilin, brazilin-(OMe)3, and brazilin-(OAc)3 exert the strongest biological effects in the MDA-MB-231 triple-negative breast cancer cell line, whereas in estrogen receptor-positive MCF7 cells, brazilin-(OAc)3 shows comparatively greater activity. Importantly, the observed effects were dependent on both concentration and exposure time.

Collectively, these findings highlight the therapeutic potential of these compounds and support continued investigation of their biological activity, particularly by identifying additional molecular targets involved in tumor progression, which may represent promising candidates for breast cancer treatment strategies.

Acknowledgments: Alberto Hernandez-Moreno, Dania A. Nava-Tapia, and Miriam D. Zuñiga-Eulogio were supported by the CONAHCYT Predoctoral Training Grant.

Conflict of Interest: None

Financial Support: This work was supported by grants from COCYTIEG awarded to J.B.-M. and SEP-PROMEP/103.5/14/11118 (UAGro-PTC-053) and SEP-CONACYT CB-2014-01-239870 awarded to N.N.-T.

Ethics Statement: None

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