




# Synthesis of novel derivatives from machilin C/D as antiproliferative agents against triple negative breast cancer

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## ABSTRACT

Lignans are small polyphenolic compounds that play an active role in plant defense against pathogens and predators. Recently, lignan machilin D was reported to be effective as an anti-tumorigenic agent in triple negative breast cancer (TNBC) tumor-bearing mice. Previous studies have relied on plant-extracted material limiting scalability and diversification of the natural scaffold. Herein, we describe a generalizable one-pot synthesis of machilin D and its derivatives via an iron chloride-induced dimerization of isoeugenol. Employing this synthetic methodology allowed for a robust diversification campaign to access seven (7) new lignan derivatives of the machilin D family with superior anti-proliferative properties in 2D and 3D TNBC models. Overall, this work enables lignan natural product-based drug discovery as a platform to identify new probes to elucidate lignan targets in biology and therapeutics for aggressive cancers such as TNBC.

## 1. Introduction

Lignans are naturally occurring phenolic dimers and trimers, which share common monomers with lignin, a rigid structural component of plant cell walls [1]. Although bioactive properties of lignans have been reported and implicated in traditional medicine, a systematic lignan-based natural product probe/drug discovery campaign is non-existent [2,3]. The historical role of natural products (NPs) for use as therapeutic agents against cancer and infectious diseases is significant [4]. Previous NP scaffolds have identified distinct biological targets and treatment options with uniquely tolerable and effective properties [5]. The structural, stereochemical complexity and diversity of natural products presents innovative opportunities for disease treatment, but not without challenges. The reduction of natural product drug discovery campaigns is associated with insufficient natural product material for isolation, physicochemical barriers, laborious structure-activity relationship (SAR) for lead optimization, difficulty with dissecting mechanism of action, and hurdles with intellectual property [6–10].

Leveraging streamlined diversification synthetic strategies and small molecule natural products, such as lignans, has the potential to transform NP drug discovery.

Traditional Chinese medicine is used as an alternative or complementary treatment for various cancers and diseases, boasting reduced side effects and prolonged survival times [11]. Although it is not recommended as the primary course of treatment, there is mounting evidence to support elements of efficacy, specifically with regards to Chinese herbal medicine [12]. Plants that have been used medicinally for centuries are under scrutiny to pinpoint their active ingredients and maximize their potential. One such plant is the Asian lizard's tail (*Saururus chinensis*), a flowering herb whose leaves have been used for centuries to treat various inflammatory conditions [13]. A recent publication by Zhen et al. isolated the lignan compound machilin D from this plant and determined it to be cytotoxic against breast cancer stem cells and well tolerated in cancer mouse models [14]. This adds to a host of evidence that this compound, and its threomer counterpart machilin C, possess biological activity [15–17], and highlights a novel treatment

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for TNBC.

Machilins C & D were first identified and named by Shimomura et al. in 1987 as components of the extract from *Machilus thunbergii*, the Japanese bay tree (Fig. 1) [18]. Since that initial study, several additional reports have been published exalting the diverse therapeutic benefits of both compounds as well as whole extracts from both *Saururus chinensis* and *Machilus thunbergii*. These agents have been investigated for the treatment of diabetes [19] and Alzheimer's Disease [20], promotion of vasorelaxation [21,22], anti-oxidant properties [15,23,24], and anti-inflammatory effects [14,17,25]. Current studies have posited machilins C and D as anticancer agents in the treatment of stomach [16], oral [26], breast [14], and blood [27,28] cancers. Potential mechanisms of action include promotion of apoptosis and autophagy [26], inhibition of DNA topoisomerases I and II [29], prevention of nitric oxide production [27,28], and blocking of interleukin IL-6 and IL-8 signaling [14].

Triple negative breast cancer (TNBC) is unique from other breast cancers in that it lacks expression of three receptors: estrogen, progesterone, and HER2 [30]. In non-TNBCs, these receptors are leveraged as drug targets to develop selective treatments with reduced side effects [31–33]. The absence of these targets relegates TNBC treatment to conventional therapies including chemotherapy, radiation, and tumor/tissue resection [34]. Although mostly effective, these treatments exhibit devastating side effects that greatly diminish quality of life. Therefore, it is imperative that novel therapies for TNBC are developed and unique targets are identified [35].

In this report, we hypothesized that diversification of machilin C/D will yield optimized lignans with superior anticancer activity to guide lignan NP drug discovery. To this end, we developed a streamlined synthetic protocol to generate lignan-based drug candidates as anti-TNBC agents and identify their SAR. Evaluation of these compounds in 2D monolayer and 3D mammosphere assays shows promising anticancer potential against TNBC. We posit that this approach will stimulate NP drug discovery efforts based on lignans and probes to identify new lignan associated targets.

## 2. Results and discussion

### 2.1. Rationale and approach

The primary obstacle to further expanding lignan-derived bioactive agents is natural product extraction yield. Prior and recent publications

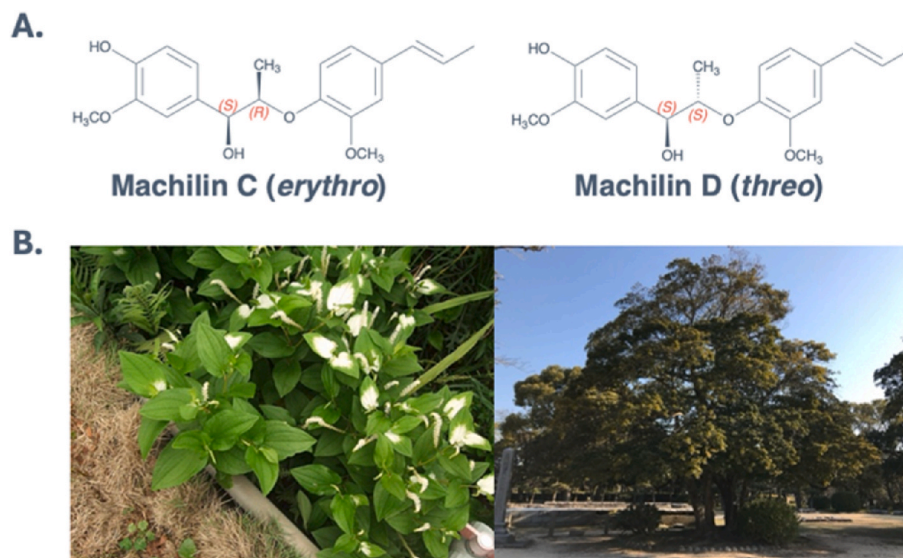
have relied on dismal yields of plant-extracted material to perform characterization and biological studies (Table 1). Although machilin C and D show great promise as novel therapeutic agents, relying on natural source extracts is an untenable path forward. A synthetic method is essential for improving scalability and elevating this novel scaffold as a drug candidate.

To our knowledge, there is only one previously reported synthetic method towards the production of either machilin C or D for biomimicry or synthetic purposes. In 2010, Xia et al. published a twelve-step asymmetric total synthesis of machilin C with 45–95 % yield at each step [39]. While stereoselective, this method lacks efficiency and scalability, rendering it unsustainable for further development. We have developed a convenient one-hour, one-pot synthesis allowing for the rapid production of machilin C&D and novel analogs of this promising scaffold. This method uses another natural product, isoeugenol, as the feedstock. Isoeugenol is used worldwide as an essential oil in the manufacturing of laundry and cleaning products, perfumes, cosmetics, and foods [40]. Isoeugenol is an abundant feedstock and can be easily synthesized from a bounty of other natural materials [41,42]. It also has the potential to be produced from lignin, a ubiquitous biopolymer in need of valorization [43]. Utilization of isoeugenol positions this method as economical, scalable, and accessible to worldwide markets.

The dimerization of isoeugenol to synthesize another lignan, dehydrodiisoeugenol (licarin A), has been thoroughly reported and the mechanism is well understood (Scheme 1) [44–46]. However, none of these sources have recognized the secondary products obtained from this synthesis: machilin C&D. We have leveraged this synthetic method and penultimate cationic site to both recreate the natural products and develop novel compounds based on the dimer scaffold. It is our goal to improve upon the cytotoxicity and anti-tumor properties previously reported while advancing an efficient synthetic method. In this manuscript, we report on the initial synthetic library, cytotoxic and anti-mammosphere properties, and suggest avenues for continued work on this promising new scaffold.

### 2.2. Synthesis and characterization

The natural products machilin C (threomer) and machilin D (erythromer) (1) were synthesized under mild conditions by combining aqueous iron (III) chloride with isoeugenol in acetone at room temperature (Fig. 2A). Similarly, the novel analogs were synthesized using our established protocol for the synthesis of machilin C/D with the addition

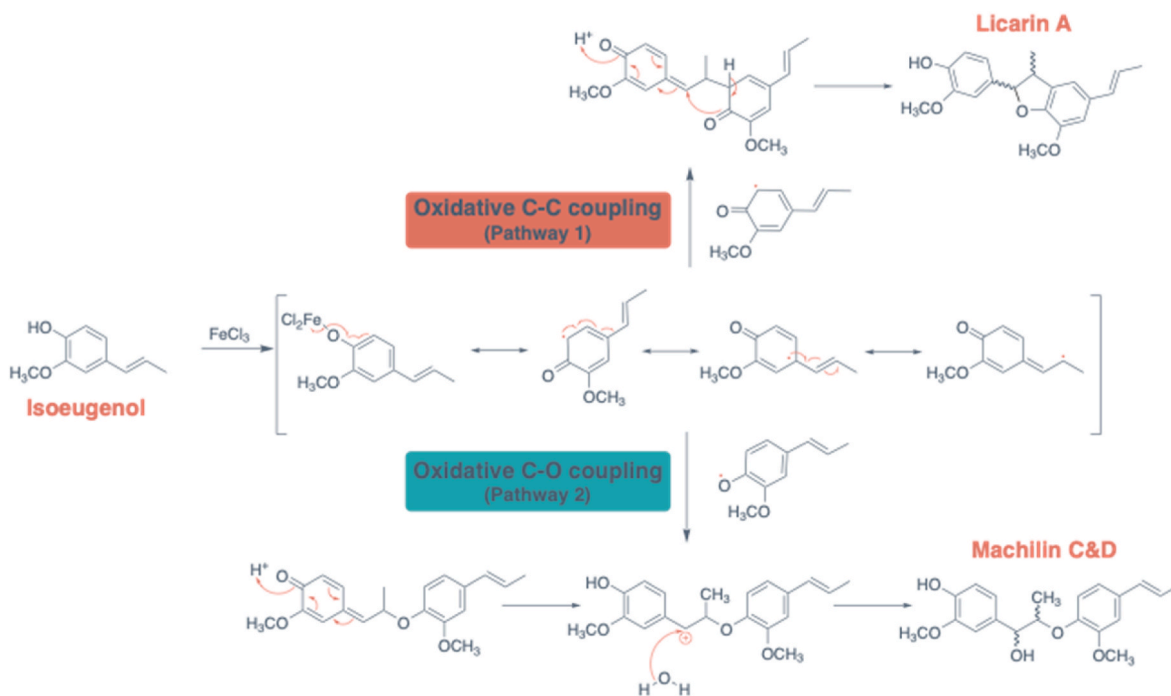


**Fig. 1.** (A) Structures of machilin C&D. May also present as [R,S] and [R,R], respectively. (B) Photographs of common machilin C&D sources: *Saururus chinensis* (left) [36] and *Machilus thunbergii* (right) [37].

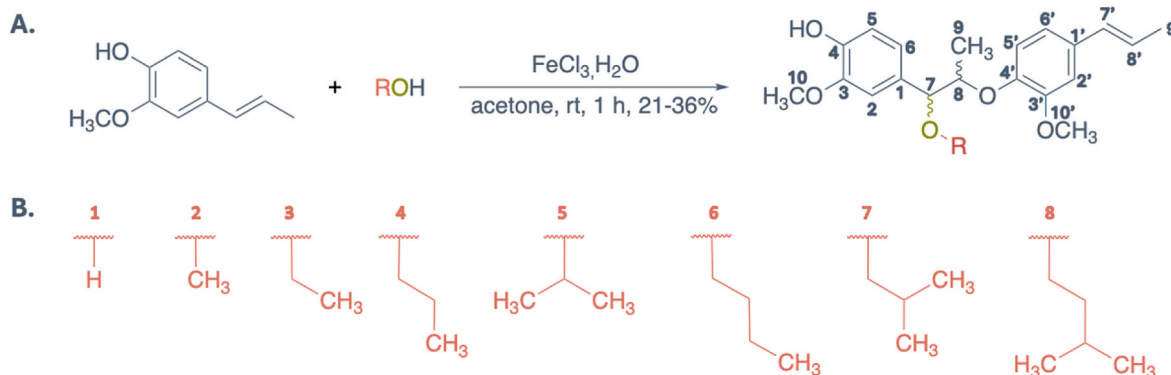
**Table 1**  
Summary of published natural sources of machilin C&D and reported extraction yields.

Plant Species	Source	Bulk Material Mass	Machilin C/D Extracted Mass <sup>a</sup>	Extraction %Yield	Reference
<i>Machilus thunbergii</i>	Bark	5 kg	14.1 mg	0.000282 %	[18]
<i>Saururus chinensis</i>	Roots	2 kg	15 mg	0.000750 %	[15]
<i>Saururus chinensis</i>	Roots	1 kg	48 mg	0.004800 %	[24]
<i>Saururus chinensis</i>	Leaves	50 g	0.07 mg	0.00014 %	[16]
<i>Myristica fragrans</i>	Aril	24 kg	8.2 mg	0.0000341 %	[38]

<sup>a</sup> If both machilin C and D were isolated, their masses were combined for this table's purpose.



**Scheme 1.** Mechanism of iron(III) chloride dimerization of isoeugenol. Top route leads to recognized product dehydrodiisoeugenol (licarin A). Bottom route is proposed mechanism for formation of machilin C&D. Image created in ChemDraw.

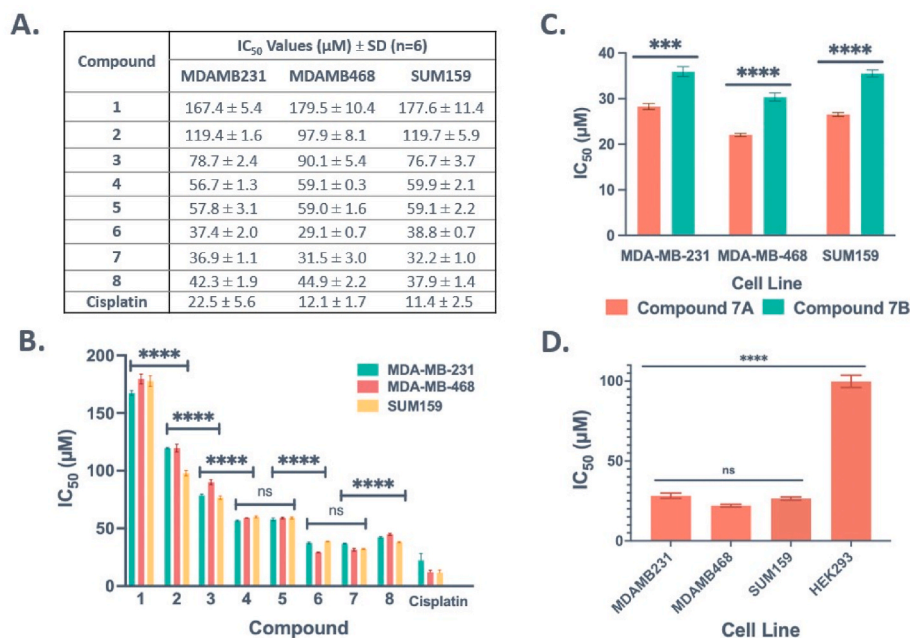


**Fig. 2.** Synthesis of lignan derivatives of machilin C/D. (A) Synthetic scheme for dimerization of isoeugenol to produce compounds 1–8. (B) Functional groups specific to compounds 1–8, labeled as such.

of methanol (2), ethanol (3), propanol (4), isopropanol (5), butanol (6), isobutanol (7), or isopentanol (8) (Fig. 3B). All products were obtained in a 21–36% product yield. Compounds 1–8 were characterized by <sup>1</sup>H and <sup>13</sup>C spectroscopy (Figs. S1–S16), high resolution mass spectrometry (Figs. S17–24), and the purity was confirmed by HPLC (Figs. S25–32).

The synthetic method is not stereoselective and produces a mix of diastereomers. For preliminary cell viability assays, we proceeded with the diastereomeric mixture to evaluate the cytotoxic effect of the

compounds on cells. Therefore, each HPLC result exhibits two peaks indicative of the diastereomeric mixtures, and the NMR spectra display dual peaks for protons in range of the two chiral centers, which has been well annotated where appropriate in the supplementary information. Machilin C&D as well as similar lignans naturally exist as a diastereomeric pair in varying ratios [47–49]. Therefore, it is reasonable to investigate the two compounds first as a pair and then as separate entities once a lead compound is prioritized.



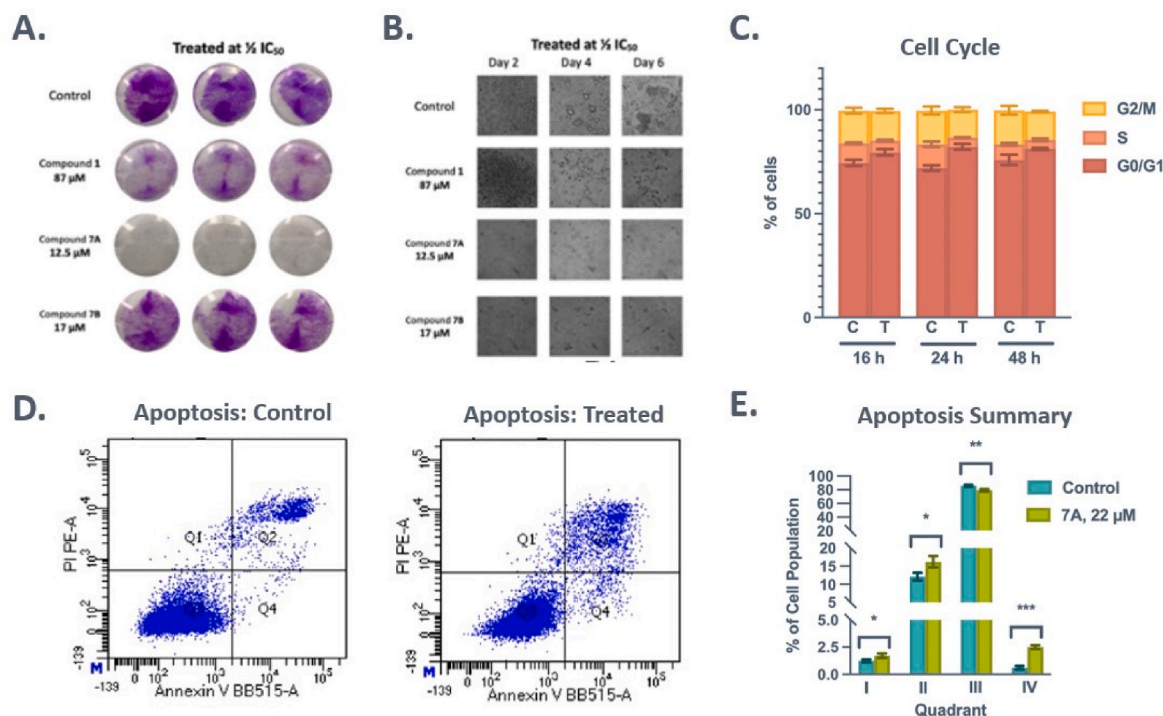
**Fig. 3.** Cell viability of machilin C/D derivatives. (A) IC<sub>50</sub> (±SEM) values as calculated by MTT assay in three TNBC cell lines. Cisplatin used at positive control. (B) Comparison chart of IC<sub>50</sub> values for compounds 1–8 and positive control cisplatin. (C) Comparison of IC<sub>50</sub> values for compounds 7A and 7B in three TNBC cell lines (\*\*P = 0.003, \*\*\*\*P < 0.0001). (D) Selectivity study of compound 7A in TNBC cell lines and normal cell line (HEK293).

Initial SAR study to assess the effect of modification at the 7-O position on biological function began with the evaluation of cell proliferation via the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) assay in three TNBC cell lines: MDA-MB-468, MDA-MB-231, and SUM159 following a 24 h exposure to compounds 1–8 with cisplatin used as a positive control (Fig. 3A). Machilin C&D exhibited IC<sub>50</sub> values of 167.4–179.5 μM, comparable to that reported by Zheng et al. [14] With extension of the alkyl chain at the 7-O position, IC<sub>50</sub> values continue to decrease (Fig. 3B) and reach a plateau with compounds 6–8. It is hypothesized that this SAR could be due to increased lipophilicity as the carbon chain lengthens. Higher lipophilicity values typically improve cell membrane penetration, allowing for better absorption [50]. It is also feasible that this modification aids in binding to the target protein(s), a theory that can be further investigated after confirmation of targets. While the IC<sub>50</sub> value remains higher than the cisplatin, this simple SAR study proves that scaffold modifications can greatly impact cytotoxicity, giving motivation for further library expansion.

Whereas previous studies have investigated the *threo*- and *erythro*-versions of this dimer scaffold (machilin C and D respectively), none have performed direct comparison studies to investigate how the stereochemical configuration impacts biological activity. Compound 7 was chosen for this investigation due to its relatively low IC<sub>50</sub> value. The diastereomers were separated by manual silica column and each fraction was identified by HPLC spectroscopy. The first diastereomer to elute in normal phase chromatography is diastereomer A (7A) and the second is diastereomer B (7B). Note that elution order is reversed in LC chromatograms as a reverse-phase column was used. Pure representatives of each diastereomer were isolated and their stereochemical configurations were assigned using <sup>1</sup>H NMR spectroscopy and LC chromatography (Figs. S31, S41, and S42). Previous research has indicated that lignan *threomers* exhibit smaller J-coupling values and exhibit upfield shifts when compared to *erythromers*. Both of these observations support 7A as the *threomer* and 7B as the *erythromer* when examining <sup>1</sup>H spectroscopy [51]. In addition, a 2021 study by Asare et. el. found that in similar B-O-4' lignan dimers, the *erythromer* typically elutes prior to the *threomer* in reverse-phase chromatography [52]. The chromatographic results indicate the 7B elutes prior to 7A in a reverse-phase system,

confirming 7B as the *erythromer* and 7A as the *threomer*. The impact of configuration on cytotoxicity was examined using MTT assay in three TNBC cell lines (Fig. 3C). It was found that the *threomer* (compound 7A) has significantly improved cytotoxicity values over 7B in all three cell lines. Therefore, compound 7A was used to determine selectivity in TNBC cell lines and normal liver cell line, HEK293 (Fig. 3D). The results show that compound 7A is significantly selective towards the 3 TNBC cell lines with a five-fold increase in potency when compared to the normal cell line, HEK293.

To further characterize the improved bioactivity of compounds 7A and 7B to compound 1, colonogenic and mammosphere formation assays were performed in TNBC cell line SUM159 at full and half IC<sub>50</sub> values (Fig. 4A and B). The results confirm that machilin C&D (compound 1) possess the potential to slow colony formation and mammosphere growth. The unlimited division of cancer cells to form colonies characterizes its tumorigenic potential. Using colony formation assay, we evaluated the antiproliferative effects of 7A in SUM159 cells. We found that colony formation was inhibited across the 7A treatment groups at half the IC<sub>50</sub> concentration and the full IC<sub>50</sub> concentrations over a 6-day treatment period demonstrating potent antiproliferative effects of 7A compared to compound 1 as shown in Fig. 4A. Thus, supporting the development of novel analogs. Further, mammospheres are breast cells grown in a 3D-architecture to recapitulate the heterogeneity and pathology of breast cancer in patients. The translational potential of mammospheres is evidenced by resistance to therapy, enriched cancer stem cells (CSCs), and metastatic potential. We tested 7A at half and full IC<sub>50</sub> concentration and found effective inhibition of SUM159 mammosphere growth, with more enhanced inhibition by 7A compared to 1. Additionally, these results confirm the effect of diastereomeric configuration on biological activity, reaffirming that the *threomer* is the more potent isomer. This supports continuation of candidate optimization using the 7A structure as a new lead compound. Future research on this scaffold will delve into the enantiomeric aspect of the synthetic products. Current research has not investigated enantiomeric ratios in the natural distribution of Machilin C or D. However, it is hypothesized that the synthetic method produces all four enantiomers. It is necessary to isolate and investigate each enantiomer of lead compound 7A to confirm or nullify differences in potency, toxicity, and determine synthetic



**Fig. 4.** (A) Clonogenic assay images in triplicate. (B) Select mammosphere images taken over period of 6 days. (C) Summary of Cell Cycle Assay using compound 7A at 22  $\mu\text{M}$  (16, 24, 48 h). (D) Representative apoptosis charts of control and treated sample (7A at 22  $\mu\text{M}$ ) at 24 h. Full chart summary in [supporting information](#). (E) Summary of Apoptosis Assay results using compound 7A at 22  $\mu\text{M}$  (24 h).

distribution. This remains an active area of research within our laboratory.

Mechanism of action studies of compound 7A was evaluated by studying the cell death pathway. The impact of compound 7A on apoptosis was examined using dual staining fluorescence-activated cell sorting (FACS) with propidium iodide and Annexin V as stains. While treated wells indicated a slight increase in necrotic (Quadrant I) and late apoptotic cells (Quadrant II), there was a significant difference in early apoptotic cells (Quadrant III). This data is indicative of significant early apoptosis induction by 7A. To assess the impact of 7A on DNA content, a cell cycle study was performed using FACS. We found that 7A induces significant drop in the S-phase population for treated cells and an increase in the G0/G1 phase, suggestive of G0/G1 arrest which indicates that the remaining cells are halted prior to DNA replication. Future work will focus on further identifying the primary mechanism of cell death and identifying protein target(s) and pathways.

### 3. Conclusion

In conclusion, we have established a convenient, tunable synthetic method for accessing novel compounds in the machilin C/D family. Using the oxidative coupling synthetic method, we identified a more potent compound than the original natural product, improving the  $\text{IC}_{50}$  value from 179  $\mu\text{M}$  to 29  $\mu\text{M}$  in representative TNBC cell lines, establishing the machilin C/D chemical scaffold viable for therapeutic development. This improvement in potency is evidenced by enhanced activity in limiting colony formation, induction of early apoptosis, and mammosphere growth activity, yielding promising results as foundations for future *in vivo* work. Additionally, the development of this synthetic method lends itself to the generation of novel lignan probes to explore mechanisms of action and elevate lignans as the new frontier of natural product drug discovery.

This work establishes the introductory chapter for a compelling lignan scaffold in a largely unexplored field for pharmaceuticals. Importantly, the reported synthetic method is extremely customizable and will

allow for synthesis of the necessary probes, continued library expansion, and modified structures to improve pharmacokinetic properties. The initial library has improved upon the natural products and proven effective in *in vitro* studies, supporting a preliminary SAR. This study will continue with target identification, structural optimization, and *in vivo* studies to support the development of these novel compounds as drug candidates.

### CRedit authorship contribution statement

**Alyson M. Ackerman:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Chibuzor Olewele:** Methodology, Investigation. **Bert C. Lynn:** Writing – review & editing, Supervision, Resources, Conceptualization. **Samuel G. Awuah:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

### 4. Notes

The authors declare the following competing financial interest(s): A. A., B.C.L. and S.G.A. have patents pending to the University of Kentucky Research Foundation.

### Declaration of competing interest

The authors declare the following competing financial interest(s): The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Samuel G. Awuah has patents pending to University of Kentucky Research Foundation.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejmc.2025.100272>.

## Data availability

Data will be made available on request.

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