

REVIEW ARTICLE

Anticancer potential of phytochemicals in canine mammary gland tumors: Molecular mechanisms, therapeutic synergy, safety concerns, and translational challenges

Kanjana Assawasuparek 

Department of Preclinic and Applied Animal Science, Faculty of Veterinary Science, Mahidol University, Salaya, Phutthamonthon, Nakhon Pathom 73170, Thailand.

ABSTRACT

Canine mammary gland tumors (CMTs) are among the most important neoplasms in female dogs and provide a valuable spontaneous model for comparative oncology. Despite advances in surgery and chemotherapy, treatment of advanced and metastatic CMTs remains limited by therapeutic resistance, systemic toxicity, and poor clinical outcomes. Phytochemicals and standardized botanical extracts have therefore attracted interest as multitarget anticancer agents and potential chemosensitizers. This review critically evaluates their molecular mechanisms, therapeutic synergy, safety, and translational potential in CMTs. Peer-reviewed literature published from January 2014 to August 2026 was identified through PubMed, Web of Science, and Google Scholar using a systematic search framework. Of 342 records identified, 254 unique records were screened, 36 full-text articles were assessed, and 10 primary experimental studies met the inclusion criteria. These studies evaluated six single-molecule phytochemicals and four standardized botanical extracts across CMT cell lines and preclinical *in vivo* models. Caspase-dependent apoptosis and modulation of the mitochondrial Bcl-2/Bax axis were the most consistently demonstrated anticancer mechanisms, while phase-specific cell cycle arrest, autophagic flux modulation, and suppression of oncogenic signaling provided additional therapeutic targets. Celastrol showed the greatest *in vitro* potency, with a half-maximal inhibitory concentration (IC₅₀) below 1.3 μ M, whereas esculetin required substantially higher concentrations and had a narrower safety margin. Combination strategies were particularly promising; curcumin with cyclophosphamide produced strong synergistic effects and enabled substantial cytotoxic dose reduction, while resveratrol combined with *Viscum album* extract demonstrated complementary anticancer activity. Conversely, indole-3-carbinol reduced primary tumor proliferation but paradoxically increased tumor embolization and visceral metastasis *in vivo*, emphasizing the need for rigorous safety assessment. Clinical translation remains constrained by poor oral bioavailability, rapid metabolism, inadequate canine-specific pharmacokinetic (PK) data, formulation variability, and the absence of prospective trials in dogs with spontaneous CMTs. Future research should prioritize standardized formulations, advanced drug delivery systems, canine PK and safety studies, and biomarker-driven clinical trials to determine whether promising phytochemicals can be translated into safe, effective adjunctive therapies for canine mammary oncology.

Keywords: anticancer phytochemicals, apoptosis, botanical extracts, canine mammary gland tumors, comparative oncology, pharmacokinetics, therapeutic synergy, translational oncology.

INTRODUCTION

Canine mammary gland tumors (CMTs) are the most frequently diagnosed neoplasms in intact female dogs, predominantly affecting middle-aged to older dogs between 6 and 8 years of age [1, 2] and accounting for 50%–70% of all tumors diagnosed in intact female dogs [3–5].

Global veterinary epidemiological registries indicate that benign lesions account for approximately 16.8% of cases, whereas aggressive malignant carcinomas constitute more than 50% of diagnoses, with increasing incidence associated with greater canine life expectancy and cumulative environmental exposures [3, 6, 7]. The etiology of CMTs is multifactorial and is strongly influenced by lifetime exposure to ovarian steroid hormones, particularly estrogen and progesterone, across successive estrous cycles [8–11]. Prepubertal ovariohysterectomy substantially reduces the risk of hormonally mediated tumor development [10], whereas early- and mid-life obesity is associated with poorer clinicopathological outcomes (odds ratio: 2.0–3.0) [12, 13], and purebred lineages exhibit distinct germline susceptibilities [14, 15].

Corresponding Author: Kanjana Assawasuparek

E-mail: kanjana.ass@mahidol.ac.th

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Prognosis and survival are strongly influenced by clinical stage (World Health Organization Stages I-V based on the tumor-node-metastasis system) and histological grade (Elston and Ellis Grades I-III) [16–18]. In early localized disease (Stage I, Grade I; tumor <3 cm and negative lymph nodes), surgical excision is associated with a prolonged median survival time (MST) of more than 2 years [16, 17, 19].

Conversely, advanced disease (Stages III-IV), high histological grade (Grade III), elevated Ki-67 proliferation indices, and lymphovascular invasion markedly reduce survival and predispose affected dogs to rapid local recurrence and early pulmonary micrometastasis [18, 20–24].

Beyond their importance in veterinary oncology, CMTs are a valuable spontaneous model for human breast cancer because they share histopathological, genomic, and molecular similarities [25, 26]. CMTs recapitulate several molecular subtypes recognized in human breast cancer, including luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-enriched, and triple-negative breast cancer (TNBC) [27]. In particular, highly aggressive variants, most notably canine inflammatory mammary carcinoma (IMC), provide a spontaneous model relevant to human TNBC because of the absence of estrogen receptor (ER), progesterone receptor (PR), and HER2 expression, rapid lymphovascular embolization, prominent inflammatory stroma, and unfavorable responses to conventional therapies [28, 29]. At the genomic level, both species share recurrent driver alterations, including *TP53* aberrations, oncogenic *PIK3CA* mutations associated with dysregulation of the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway, and inherited susceptibility associated with *BRCA1* and *BRCA2* alterations [30–32]. Because companion dogs share human environments, possess intact immune systems, and spontaneously develop tumors with substantial clonal heterogeneity, canine models may overcome several translational limitations of immunodeficient rodent xenografts and provide a valuable platform for comparative oncology research [29, 33, 34].

Despite therapeutic advances, management of advanced CMTs continues to face two major translational challenges: the limited long-term effectiveness of conventional systemic therapies in aggressive disease and the failure of most preclinical phytochemical candidates to progress beyond experimental evaluation into routine veterinary practice [33, 35]. Standard-of-care (SOC) management of localized CMTs primarily relies on surgical mastectomy [3, 14, 16]. However, in advanced malignant carcinomas (Stages III-IV) and IMC, surgery alone is unlikely to provide curative treatment, necessitating consideration of adjuvant cytotoxic chemotherapy, including doxorubicin, carboplatin, cyclophosphamide, 5-fluorouracil, and docetaxel [36]. Adjuvant protocols provide modest clinical benefits, with reported MSTs of 240–350 days in advanced carcinomas receiving adjuvant systemic protocols [36] and approximately 60 days in IMC [37]. Conventional chemotherapeutic agents are also limited by dose-limiting toxicities, including grade 3/4 myelosuppression, gastrointestinal injury, and sterile hemorrhagic cystitis, as well as the emergence of multidrug resistance (MDR) mediated by adenosine triphosphate-binding cassette efflux transporters [37–41].

Evidence-based phytotherapy has emerged as a potential multitarget therapeutic strategy that can modulate several cancer-associated pathways and may help address limitations of conventional chemotherapy, including toxicity and therapeutic resistance [42–44]. Bioactive botanical agents have shown pleiotropic anticancer activities, including modulating dysregulated oncogenic signaling, downregulating Ki-67 expression, modulating MDR-associated mechanisms, and enhancing chemotherapeutic activity, including curcumin combined with cyclophosphamide and resveratrol combined with *Viscum album* [45–50]. Nevertheless, the existing phytotherapy literature remains predominantly focused on *in vitro* cytotoxicity and antiproliferative effects, with comparatively less attention to clinically important translational considerations, including bioavailability, pharmacokinetics (PK), safety, product standardization, and clinical validation [33]. In particular, free polyphenols face substantial canine-specific PK limitations, including poor aqueous solubility, low oral peak plasma concentrations (C_{max}), short elimination half-lives ($t_{1/2}$), and extensive first-pass phase II glucuronidation and sulfation, which may prevent the attainment of therapeutically active micromolar concentrations *in vivo* [51–54]. In addition, inadequate chemistry, manufacturing, and controls (CMC), batch-to-batch variability, and the near-total absence of prospective studies in client-owned dogs continue to impede clinical translation [32, 33, 55, 56].

Despite expanding experimental interest in botanical therapeutics for CMTs, the existing literature remains fragmented across disparate cell lines, unstandardized botanical preparations, diverse cellular targets, and varying exposure protocols, making direct cross-study comparisons challenging [33]. Most available studies focus narrowly on isolated *in vitro* cytotoxic potency without cross-referencing tumor selectivity, target engagement, or paradoxical *in vivo* dissemination behaviors [33, 52]. Crucially, there is currently no unified comparative framework that systematically evaluates single-molecule phytochemicals alongside standardized botanical

extracts across defined intracellular signaling axes while reconciling experimental active concentrations with translational feasibility and safety margins [33, 35]. Addressing this gap through an integrated, multi-parameter synthesis is essential to prioritize promising candidates and rational combination regimens for advanced formulation development and biomarker

Therefore, this structured narrative review critically synthesizes peer-reviewed literature published from January 2014 to August 2026 on phytochemicals and standardized botanical extracts investigated for CMTs. The review focuses on six single-molecule phytochemicals, celastrol, esculetin, berberine, curcumin, resveratrol, and indole-3-carbinol, and four standardized botanical extracts derived from *Gynura procumbens*, *Calotropis procera*, *Euphorbia royleana*, and *Polytrichastrum alpinum*, evaluated across CMT cell lines and preclinical *in vivo* models. Specifically, the review examines their molecular mechanisms, including caspase-dependent apoptosis, Bcl-2/Bax-mediated mitochondrial regulation, cell cycle arrest, autophagic flux modulation, and therapeutic synergy; compares their potency and safety characteristics; and critically evaluates the relationship between experimentally effective concentrations and their translational feasibility.

In addition, this review aimed to identify major barriers to clinical translation, including poor bioavailability, inadequate canine-specific PK information, formulation and CMC limitations, potential adverse or paradoxical *in vivo* effects, and the scarcity of prospective studies in dogs with spontaneous CMTs. By integrating mechanistic evidence with pharmacological and translational considerations, the review seeks to establish an evidence-based framework for prioritizing promising phytochemical candidates and combination strategies and to define future research priorities involving standardized formulations, advanced drug delivery approaches, canine PK and safety studies, and biomarker-driven clinical trials within a One Health comparative oncology framework.

REVIEW METHODOLOGY

Study design and review classification

This review used a structured narrative synthesis with quantitative data extraction to improve transparency, reproducibility, and scientific rigor. It was not classified as a formal systematic review or meta-analysis because of substantial heterogeneity among the included *in vitro* cell models and preclinical experimental protocols. Nevertheless, literature identification and study selection followed a predefined systematic search framework. Formal risk-of-bias assessment tools, such as Cochrane RoB 2 or the Systematic Review Center for Laboratory Animal Experimentation (SYRCLE) risk-of-bias tool, were not applied. Instead, the methodological quality of eligible primary studies was qualitatively appraised according to the use of appropriate vehicle and/or positive controls, biological replication, reporting of statistical power, and authentication or documentation of the origin of canine mammary tumor cell lines.

Search strategy and database syntax

Comprehensive electronic literature searches were initially conducted in January 2024 and systematically updated on August 15, 2026, across PubMed, Web of Science Core Collection, and Google Scholar for studies published from January 2014 to August 2026.

Search strategies incorporated Medical Subject Headings (MeSH), where applicable, together with free-text terms adapted to the syntax of each database.

PubMed: (canine mammary neoplasms [MeSH] OR canine mammary tumor [Title/Abstract] OR canine mammary gland carcinoma [Title/Abstract] OR CMT [Title/Abstract]) AND (biological products [MeSH] OR phytochemicals [MeSH] OR plant extracts [Title/Abstract] OR celastrol [Title/Abstract] OR esculetin [Title/Abstract] OR berberine [Title/Abstract] OR curcumin [Title/Abstract] OR resveratrol [Title/Abstract] OR indole-3-carbinol [Title/Abstract] OR *Gynura procumbens* [Title/Abstract] OR *Calotropis procera* [Title/Abstract] OR *Euphorbia royleana* [Title/Abstract] OR *Polytrichastrum alpinum* [Title/Abstract]) AND (apoptosis [MeSH] OR caspases [MeSH] OR Bcl-2 [Title/Abstract] OR cell cycle arrest [Title/Abstract] OR autophagy [MeSH] OR drug synergism [MeSH]).

Web of Science: TS = (canine mammary tumor OR canine mammary gland carcinoma OR CMT) AND TS = (phytochemicals OR natural compounds OR botanical extracts OR celastrol OR esculetin OR berberine OR curcumin OR resveratrol OR indole-3-carbinol OR *Gynura procumbens* OR *Calotropis procera* OR *Euphorbia royleana* OR *Polytrichastrum alpinum*) AND TS = (apoptosis OR caspase OR Bcl-2 OR cell cycle arrest OR autophagy OR synergy OR combination therapy).

Google Scholar: allintitle: (canine mammary OR CMT) (phytochemical OR extract OR curcumin OR resveratrol OR celastrol OR esculetin OR berberine).

Study selection, screening, and eligibility criteria

A single investigator (KA) performed literature screening and data extraction using predefined eligibility criteria and a standardized data extraction protocol to maintain methodological consistency. The initial electronic searches identified 342 records. After removal of 88 duplicate records, 254 unique records underwent title and abstract screening. Of these, 218 were excluded because they were irrelevant to the predefined scope, including studies involving non-canine species, non-mammary neoplasms, synthetic non-phytochemical therapeutics, or general narrative reviews. The remaining 36 full-text articles were assessed for eligibility.

Studies were eligible if they (1) evaluated defined single-molecule phytochemicals or standardized botanical extracts using established canine mammary tumor cell lines or relevant *in vivo* models and (2) investigated defined intracellular signaling pathways, quantitative cytotoxicity indices such as the IC_{50} , or quantitative drug interaction parameters such as the combination index (CI). Studies evaluating uncharacterized crude extracts without defined phytochemical markers or lacking intracellular mechanistic or quantitative potency endpoints were excluded. Following full-text assessment, 26 studies were excluded, including 14 because of uncharacterized crude extract composition and 12 because of the absence of defined intracellular mechanistic or potency endpoints. Consequently, 10 primary experimental studies met the predefined eligibility criteria and were included in the qualitative and quantitative synthesis. The study identification, screening, eligibility assessment, and inclusion process is presented in Figure 1 [57]. As a methodological limitation, literature screening, eligibility assessment, and data extraction were conducted by a single investigator (KA) without independent duplicate verification, which may introduce selection bias.

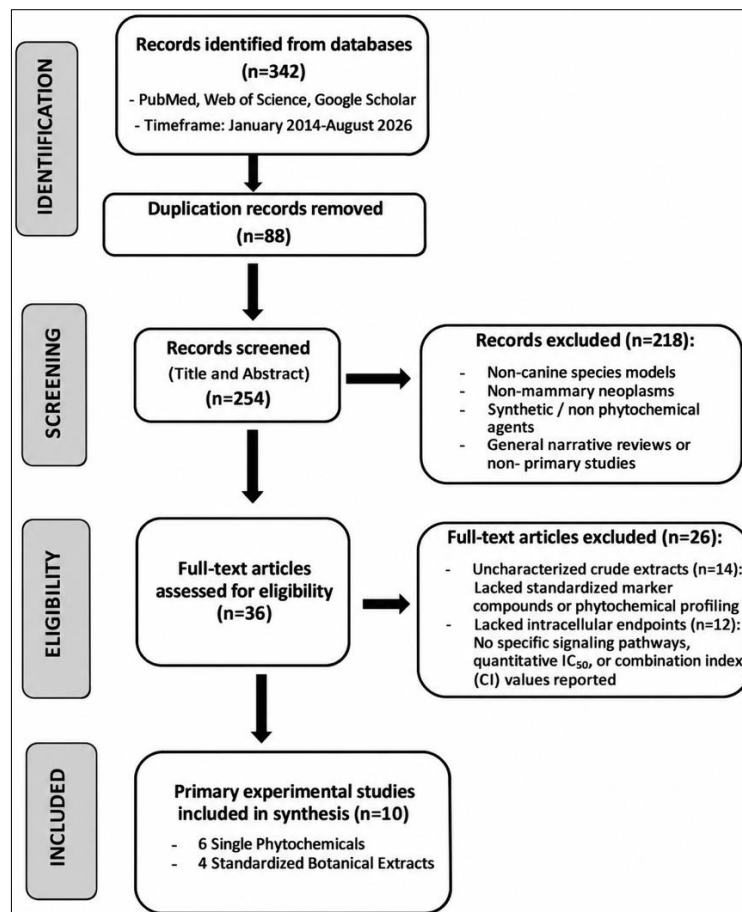


Figure 1: Literature search and study selection flow diagram.

Data extraction and synthesis

Quantitative and qualitative data from eligible studies were extracted using a standardized framework and organized into comparative summaries (Tables 1–4). Extracted variables included canine mammary tumor cell line characteristics; molecular subtype and receptor status (where reported); phytochemical or botanical extract standardization characteristics; quantitative effect measures; IC_{50} values; apoptotic fractions; cell cycle distribution changes; and CI values derived using the Chou-Talalay method. Reported *in vivo* pharmacokinetic, tumor progression, and metastatic endpoints were also extracted where available. Given the methodological

heterogeneity among the included experimental systems, the extracted evidence was synthesized comparatively rather than subjected to formal meta-analysis.

Translational appraisal framework

To objectively evaluate the clinical applicability of the investigated agents, an author-defined translational appraisal framework was applied. Each phytochemical or botanical extract was assigned a translational readiness score based on predefined criteria:

Green (High translational potential): Agents demonstrating strongly validated synergistic combination effects, favorable safety profiles, and/or supported by canine clinical evidence.

Amber (Moderate translational feasibility): Promising preclinical candidates whose clinical translation is currently constrained by pharmacokinetic limitations, requirement of high/millimolar effective concentrations, narrow safety margins, insufficient formulation standardization, or a lack of *in vivo* canine data.

Red (Critical precaution): Agents associated with paradoxical adverse *in vivo* findings (e.g., increased metastatic dissemination) that require rigorous safety investigation before any clinical translation can be considered.

Flow diagram illustrating literature identification, screening, eligibility assessment, and study inclusion, adapted from standard reporting guidelines [57]. Searches of PubMed, Web of Science, and Google Scholar for literature published from January 2014 to August 2026 identified 342 records

After removal of duplicates ($n = 88$), 254 unique records underwent title and abstract screening, of which 218 were excluded. The remaining 36 full-text articles were assessed for eligibility, and 26 were excluded because of uncharacterized crude extract composition ($n = 14$) or the absence of defined intracellular mechanistic or potency endpoints ($n = 12$). Ultimately, 10 primary experimental studies evaluating six single-molecule phytochemicals and four standardized botanical extracts met the predefined eligibility criteria and were included in the qualitative and quantitative synthesis [47, 48, 51, 52, 58–63].

MOLECULAR MECHANISMS AND COMPARATIVE PATHWAY DYNAMICS OF PHYTOCHEMICALS IN CANINE MAMMARY TUMORS

The antineoplastic effects of phytochemicals and botanical extracts in CMTs extend beyond nonspecific cytotoxicity and involve modulation of distinct, highly conserved intracellular signaling networks. Rather than functioning solely as empirical cytotoxic agents, bioactive phytochemicals can target specific regulatory checkpoints involved in programmed cell death, mitochondrial membrane integrity, cell cycle progression, autophagic flux, and oncogenic signaling [64, 65]. However, critical evaluation of the available evidence reveals several methodological and translational limitations that must be considered before these preclinical findings can inform veterinary clinical oncology.

First, most reported molecular mechanisms come from single-laboratory investigations using isolated *in vitro* cell line monocultures, with limited independent replication across laboratories and little validation in spontaneous tumors from immunocompetent canine patients. Consequently, the reproducibility and clinical relevance of several proposed mechanisms remain insufficiently established. Second, a substantial disparity exists between concentrations demonstrating efficacy *in vitro* and those likely to be achievable *in vivo*. Several agents exert measurable anticancer effects only at high micromolar or millimolar concentrations, which may be difficult to achieve or sustain in canine plasma because of rapid metabolic clearance, limited bioavailability, or dose-limiting toxicity. Third, although tumor selectivity has been evaluated for selected compounds using non-tumorigenic canine cell lines, including Madin-Darby canine kidney (MDCK) cells and canine aortic endothelial cells (CnAOECs), comprehensive selectivity indices (SI) remain unavailable for many candidate compounds. Furthermore, evaluations of cancer stem cell markers, epithelial-mesenchymal transition, and orthogonal molecular validation using complementary approaches, such as quantitative real-time polymerase chain reaction, Western blotting, and immunofluorescence, remain limited.

Collectively, these limitations indicate that mechanistic activity demonstrated under controlled *in vitro* conditions should not be interpreted as direct evidence of clinical efficacy. Comparative assessment should therefore consider not only molecular targets and cytotoxic potency but also tumor selectivity, achievable *in vivo* exposure, safety, reproducibility, and the extent of biological validation. To facilitate this comparison, the biological characteristics, receptor status, molecular subtype, experimental context, and translational readiness of the evaluated CMT cell models, including multidrug-resistant CMT-7364 [66], spindle-cell CMT-U309 [67], and metastatic lineages [68, 69], are summarized in Table 1 [47, 48, 51, 58–62].

Table 1: Biological characteristics, receptor status, and molecular subtype classifications of canine mammary tumor and normal control cell lines.

Cell line	Histopathological classification	ER status	PR status	HER2 status	Molecular subtype and genetic profile	Evaluated phytochemicals and experimental context	Translational readiness score	References
CMT-7364	Canine mammary carcinoma (intraductal papillary carcinoma origin)	Negative	Negative	Negative	Triple-negative (TNBC-like)	Celastrol: Evaluated for low-micromolar p53-independent caspase-3/9-mediated apoptosis and G2/M mitotic arrest	Amber (high <i>in vitro</i> potency [$IC_{50} < 1.3 \mu M$], but constrained by poor solubility and lack of canine PK data)	[58, 66]
CIPp	Primary canine inflammatory mammary carcinoma	Negative	Negative	Negative	Triple-negative/basal-like	Celastrol: Evaluated for dose-dependent inhibition of proliferation, migration, and invasion	Amber (potent <i>in vitro</i> antimetastatic activity; lacks <i>in vivo</i> canine PK and safety data)	[58]
CMT-U27	Canine mammary carcinoma	Not reported	Not reported	Not reported	<i>TP53</i> -altered cell line	Esculetin and curcumin + cyclophosphamide: Lineage-specific G0/G1 arrest by esculetin; strong synergy with cyclophosphamide (CI = 0.29)	Green for curcumin combination (CI < 0.5; >50% chemotherapy dose reduction); Amber for esculetin (millimolar concentrations required)	[47, 59]
CF41.Mg	Canine mammary adenocarcinoma	Not reported	Not reported	Not reported	Receptor status not consistently reported	Esculetin, berberine, and <i>Calotropis procera</i> root extract: Evaluated for Bcl-2 suppression, S-phase delay, and G0/G1 arrest	Amber (requires high concentrations [berberine, 100 μM ; esculetin, millimolar range], with low oral bioavailability and cardenolide-associated risks)	[51, 59, 60]
CHMp-13a	Primary canine complex mammary carcinoma	Not reported	Not reported	Not reported	EGFR-high phenotype	GPE: Evaluated for EGFR downregulation and caspase-3/7 activation	Amber (extract-level efficacy; requires marker standardization [quercetin/kaempferol] and <i>in vivo</i> validation)	[61]
CHMp-5b	Primary canine complex mammary carcinoma	Not reported	Not reported	Not reported	EGFR-overexpressing mammary carcinoma model	GPE: Evaluated for dose- and time-dependent cell death and antimigratory activity	Amber (promising anti-invasive activity; evidence restricted to single laboratory <i>in vitro</i> monocultures)	[61]
MGT316	Canine mammary carcinoma	Not reported	Not reported	Not reported	Malignant epithelial lineage; receptor status not reported	PAME: Evaluated for cleaved caspase-3/ PARP activation, G0/G1 arrest, and inhibition of matrix invasion	Amber (multitargeted intrinsic apoptosis and favorable epithelial safety; preclinical <i>in vitro</i> evidence only)	[62]
MGT612	Canine mammary carcinoma	Not reported	Not reported	Not reported	Malignant epithelial lineage; receptor status not reported	PAME: Evaluated for suppression of cell viability and colony formation and increased Bax/ Bcl-2 ratio	Amber (promising moss extract; requires chemical production scale-up and canine PK evaluation)	[62]
CMT-U309	Canine spindle-cell mammary carcinoma	Negative	Positive	Not reported	Spindle-cell phenotype	Curcumin + cyclophosphamide: Chou-Talalay combination analysis demonstrated synergy (CI = 0.42; DRI > 2.0)	Green (strong synergy and 50% chemotherapy dose reduction; potential for nano-curcumin development)	[47, 67]

Cell line	Histopathological classification	ER status	PR status	HER2 status	Molecular subtype and genetic profile	Evaluated phytochemicals and experimental context	Translational readiness score	References
UNESP-CM9	Primary canine mammary carcinoma	Not reported	Not reported	Not reported	Malignant epithelial lineage	Resveratrol + <i>Viscum album</i> extract: Evaluated for dose reduction and complete suppression of cell viability (0%)	Green (promising combination supported by clinical adjuvant evidence in dogs)	[48, 68, 69]
UNESP-CM60	Canine mammary carcinoma	Not reported	Not reported	Not reported	Not reported	Resveratrol + <i>V. album</i> extract: Evaluated for inhibition of scratch-wound closure (2.41% vs. 39.46% in controls)	Green (marked antimigratory activity <i>in vitro</i> with reported systemic immune effects <i>in vivo</i>)	[48, 68, 69]
MDCK	Normal canine kidney epithelium	N/A	N/A	N/A	Non-tumorigenic epithelial control	Celastrol: Non-malignant control used to assess SI and tumor-selective cytotoxicity	N/A (non-neoplastic control cell line)	[58]
CnAOECs	Normal canine aortic endothelial cells	N/A	N/A	N/A	Non-tumorigenic endothelial control	Esculetin: Normal vascular endothelial control used to assess nonselective endothelial cytotoxicity at 1.0 mM	N/A (non-neoplastic control cell line)	[59]

Receptor status and molecular subtype classifications are based on the primary experimental literature; unavailable parameters are designated as “Not reported.” Translational readiness score: Green = higher translational potential or demonstrated synergistic potential; Amber = promising preclinical candidate constrained by pharmacokinetic limitations, millimolar concentration requirements, or narrow safety margins; Red = critical safety concern, paradoxical prometastatic risk, or clinically contraindicated as monotherapy. Bax = Bcl-2-associated X protein; Bcl-2 = B-cell lymphoma 2; CI = combination index; CMT = canine mammary gland tumor; CnAOECs = canine aortic endothelial cells; DRI = dose reduction index; EGFR = epidermal growth factor receptor; ER = estrogen receptor; GPE = *Gynura procumbens* leaf extract; HER2 = human epidermal growth factor receptor 2; IC₅₀ = half-maximal inhibitory concentration; MDCK = Madin-Darby canine kidney; N/A = not applicable; PAME = *Polytrichastrum alpinum* methanol extract; PARP = poly(ADP-ribose) polymerase; PK = pharmacokinetics; PR = progesterone receptor; SI = selectivity index; TNBC = triple-negative breast cancer.

APOPTOSIS INDUCTION AND CASPASE CASCADE ACTIVATION

Apoptosis represents the most extensively validated antineoplastic endpoint across the included CMT literature and is characterized by proteolytic activation of initiator caspases (caspase-8 and caspase-9) and executioner caspases (caspase-3 and caspase-7), downstream cleavage of poly(ADP-ribose) polymerase (PARP), and apoptotic DNA fragmentation [64, 65]. Of the 10 included experimental studies, eight demonstrated apoptosis induction, although substantial differences were observed in potency, tumor selectivity, and cell line-specific responses.

Celastrol (*Tripterygium wilfordii*)

Celastrol, a pentacyclic triterpenoid isolated from *T. wilfordii* [70, 71], is among the most potent single-molecule phytochemicals investigated in CMT models. In the canine mammary carcinoma cell lines CMT-7364 [66] and CIPp, celastrol produced pronounced dose- and time-dependent inhibition of cell viability and induction of apoptosis at low-micromolar concentrations [58]. In CMT-7364 cells, the IC₅₀ values were 1.236 μ M at 24 h and 1.007 μ M at 48 h ($p < 0.01$), whereas the corresponding values in CIPp cells were 1.156 μ M and 0.968 μ M, respectively ($p < 0.01$) [58].

At concentrations increasing to 1.6 μ M, celastrol induced a near-complete loss of cell viability, accompanied by marked increases in cleaved caspase-3 and cleaved caspase-9, supporting activation of caspase-mediated apoptosis [58]. Importantly, celastrol exhibited lower cytotoxicity toward non-tumorigenic MDCK cells than toward malignant cells, suggesting a degree of tumor selectivity. Aggressive CMT models frequently harbor *TP53* alterations and impaired p53 signaling, which may contribute to treatment resistance and malignant progression [31]. Celastrol induced apoptosis and increased p21 and p27 expression independently of functional upstream p53 signaling, suggesting a potential mechanism for bypassing p53-associated resistance. However, evidence for celastrol in CMTs is currently derived from a single mechanistic study from one laboratory, emphasizing the need for independent validation and robust *in vivo* investigations [58]. Furthermore, its translational development is constrained by poor aqueous solubility, limited bioavailability, and a narrow systemic therapeutic window in mammals.

Esculetin (6,7-dihydroxycoumarin)

Esculetin, a naturally occurring coumarin derivative found in *Fraxinus rhynchophylla*, *Artemisia capillaris*, and *Aesculus hippocastanum* [72, 73], induces caspase-3-mediated apoptosis and cell cycle arrest in canine mammary tumor cells. However, compared with celastrol, esculetin is less potent and requires substantially higher concentrations to achieve antitumor effects [58, 59]. Its activity also demonstrates pronounced cell line-specific variation.

CMT-U27 cells were comparatively sensitive to esculetin, with 0.5 mM exposure reducing cell viability by approximately 40% at 24 h and 80% at 48 h ($p < 0.05$) [59]. In contrast, CF41.Mg cells were more resistant, exhibiting viability reductions of approximately 20% and 35% at 24 and 48 h, respectively, under the same 0.5 mM exposure conditions ($p < 0.05$) [59].

Mechanistically, esculetin induces caspase-3 cleavage together with transcriptional downregulation of cyclin D1 and cyclin-dependent kinase 4 (CDK4) [59]. However, its effective concentration range presents an important translational limitation. Although esculetin exhibited minimal cytotoxicity toward CnAOECs at concentrations ≤ 0.5 mM, acute nonselective cytotoxicity was observed at 1.0 mM ($p < 0.01$) [59]. Thus, the millimolar concentrations required for substantial antitumor activity, together with cytotoxicity toward non-tumorigenic cells at higher concentrations, indicate a narrow experimental therapeutic window. These findings warrant investigation of formulation or targeted-delivery approaches capable of improving tumor exposure while reducing systemic toxicity.

Standardized *G. procumbens* leaf extract

G. procumbens is a medicinal plant containing diverse bioactive constituents, including the flavonols quercetin, kaempferol, and rutin; chlorogenic acid derivatives; and phenolic glycosides [74]. In the canine mammary carcinoma cell lines CHMp-13a and CHMp-5b, standardized *G. procumbens* leaf extract suppressed proliferation and wound closure activity in a dose- and time-dependent manner ($p < 0.05$) [61]. Mechanistically, the extract activated caspase-3/7-dependent apoptotic pathways together with significant downregulation of EGFR protein expression [61].

Because EGFR overexpression has been associated with aggressive biological behavior and therapeutic resistance in canine mammary carcinomas, its suppression may contribute to the observed antiproliferative and antimigratory effects of *G. procumbens* extract [61]. Nevertheless, these findings remain preclinical, and reproducible standardization of bioactive marker concentrations and batch-to-batch composition is required before its translational potential can be adequately assessed. Independent validation across additional CMT models and subsequent *in vivo* evaluation are also required.

Standardized *P. alpinum* methanol extract

P. alpinum, an extremophilic polar and alpine moss, contains bioactive phenolic constituents, including the benzonaphthoxanthrenones ohioensins F and G, which have demonstrated antioxidant activity [75]. In the canine mammary carcinoma cell lines MGT316 and MGT612, *P. alpinum* methanol extract suppressed colony formation, cell viability, and transwell extracellular matrix invasion ($p < 0.05$) [62]. Annexin V/propidium iodide staining demonstrated significant increases in early and late apoptotic cell populations, accompanied by increased levels of cleaved caspase-3 and cleaved PARP [62].

Concurrently, the extract downregulated the antiapoptotic protein Bcl-2 and upregulated the proapoptotic protein Bax, shifting the balance of Bcl-2 family proteins toward apoptosis [62]. Importantly, *P. alpinum* methanol extract exhibited negligible cytotoxicity toward the non-tumorigenic control cells evaluated in the study, suggesting a favorable *in vitro* selectivity profile [62]. However, its antineoplastic activity requires confirmation in independent experimental systems and *in vivo* CMT models before drawing conclusions about therapeutic applicability.

Mitochondrial membrane integrity and the Bcl-2/Bax rheostat

The intrinsic apoptotic pathway is regulated by mitochondrial outer membrane permeabilization (MOMP), with the balance between proapoptotic proteins, including Bax and Bak, and antiapoptotic proteins, including Bcl-2, Bcl-xL, and Mcl-1, determining susceptibility to mitochondrial apoptosis [76-78]. Disruption of this balance in favor of proapoptotic signaling facilitates mitochondrial membrane permeabilization and downstream activation of the apoptotic machinery.

***C. procera* root extract**

C. procera (Apocynaceae) root extract contains cytotoxic cardenolides, including calotropin, uscharin, and calotoxin, as well as other cardiac glycosides [79, 80]. In CF41.Mg cells, ethanolic *C. procera* root extract demonstrated significant antineoplastic activity, with an IC_{50} of 9.00 $\mu\text{g/mL}$ [60]. Immunoblot analysis showed significant downregulation of the antiapoptotic Bcl-2 protein compared with untreated controls ($p < 0.05$), supporting mitochondrial apoptosis modulation as part of its cytotoxic activity [60].

CRITICAL METHODOLOGICAL LIMITATIONS IN CURRENT LITERATURE

A major methodological limitation of the current CMT phytotherapy literature, including studies evaluating *C. procera*, is the reliance on qualitative or limited-parameter Western blot analyses. Many studies report changes in Bcl-2 expression without providing exact quantitative fold changes or mathematically derived Bax/Bcl-2 ratios. Furthermore, other antiapoptotic proteins involved in maintaining mitochondrial integrity, particularly Bcl-xL and Mcl-1, remain insufficiently investigated. Orthogonal validation integrating quantitative real-time polymerase chain reaction at the transcriptional level, Western blotting at the protein level, and confocal immunofluorescence to assess Bax mitochondrial translocation has also not been systematically applied. Consequently, although the available findings support modulation of mitochondrial apoptotic signaling, more comprehensive molecular validation is required to establish the magnitude, reproducibility, and mechanistic specificity of these effects.

Phase-specific cell cycle checkpoint-arrest and lineage sensitivity

Uncontrolled cell proliferation driven by dysregulated CDK activity is a fundamental feature of CMTs [29]. Bioactive phytochemicals may exert antineoplastic effects by disrupting aberrant cell cycle progression and inducing arrest at specific checkpoints. Flow cytometric cell cycle analysis has therefore been used to quantify treatment-induced redistribution of cell populations across the G0/G1, S, and G2/M phases.

Berberine (isoquinoline alkaloid)

Berberine is a protoberberine isoquinoline alkaloid primarily isolated from *Coptis* spp., including medicinal preparations of *Coptidis rhizoma* [81]. In CF41.Mg cells, berberine inhibited cell viability in a dose- and time-dependent manner, with significant effects reported at 100 μM ($p < 0.005$) [51]. Quantitative flow cytometric analysis showed that berberine induced G0/G1-phase arrest, increasing the proportion of cells in G0/G1 by approximately 18%- 22% compared with untreated controls ($p < 0.005$). This redistribution was accompanied by inhibition of progression into the DNA-replicating S-phase and an increase in the hypodiploid sub-G1 population, consistent with apoptotic cell death [51].

Celastrol

In CMT-7364 and CIPp cells, celastrol induced pronounced G2/M-phase arrest [58]. Flow cytometric analysis demonstrated a dose-dependent increase in the proportion of cells in G2/M following exposure to 0, 0.3, and 0.9 μM celastrol. In CMT-7364 cells, the G2/M fraction increased from 5.6% in untreated cells to 20.3% and 32.7% following exposure to 0.3 and 0.9 μM celastrol, respectively ($p < 0.01$) [58]. Similarly, in CIPp cells, the G2/M fraction increased from 12.0% to 21.9% and 32.4%, respectively ($p < 0.01$) [58].

This cell cycle arrest was accompanied by increased expression of p21 and p27 and suppression of cyclin D1 [58]. Collectively, these findings indicate that celastrol interferes with cell cycle progression and promotes accumulation of CMT cells in the G2/M phase. However, because these observations come from a limited experimental evidence base, independent mechanistic validation is required before researchers can establish the precise regulatory pathway responsible for this arrest.

Lineage-specific cell cycle arrest by esculetin

Esculetin induced different cell-cycle responses in the evaluated CMT cell lines. In CMT-U27 cells, treatment with 1 mM esculetin induced G0/G1-phase arrest, increasing the G0/G1 population from 54.2% to 68.2% ($p < 0.05$) [59]. In contrast, CF41.Mg cells exhibited an S-phase delay, with the S-phase population increasing from 24.5% to 36.5% ($p < 0.05$) [59].

These findings demonstrate cell line-specific differences in the response to esculetin and may reflect underlying biological heterogeneity among CMT models. However, the available evidence does not establish whether these differences are attributable to molecular subtype, CDK signaling, or other regulatory pathways. Further mechanistic studies are therefore required to determine the molecular basis of the divergent cell cycle responses [59].

Autophagic flux modulation: From prosurvival recycling to enhanced cytotoxicity

Autophagy has context-dependent functions in cancer. Although basal autophagy supports cellular homeostasis and organelle quality control, malignant cells may exploit autophagy to survive metabolic stress, hypoxia, and cytotoxic agents [82–84]. In malignant CMTs, reduced expression of the autophagy-associated protein Beclin-1 has been associated with aggressive clinicopathological characteristics, including higher histological grade, increased mitotic activity, extensive necrosis, and poorer overall survival [85].

E. royleana extract and autophagic flux inhibition

E. royleana contains diverse bioactive terpenoids, including ingenane- and ingol-type diterpenoids and triterpenoids, which have been associated with its biological activities [86]. Hexane and ethanol fractions of *E. royleana* demonstrated antiproliferative and colony-suppressive effects against CMT cells and significantly reduced solid tumor volume in mouse xenograft models [63].

Treatment with *E. royleana* extract increased expression of the autophagosome-associated marker LC3-II and decreased mutant p53 expression, accompanied by extensive formation of acidic vesicular organelles (AVOs), as demonstrated using acridine orange staining [63]. These findings indicate that *E. royleana* induces autophagy. Importantly, pharmacological inhibition of late-stage autophagy with bafilomycin A1 enhanced *E. royleana*-induced apoptosis, suggesting that the treatment-associated autophagic response may exert a cytoprotective function in this experimental setting [63]. Thus, inhibition of autophagic flux may enhance extract-mediated cytotoxicity. However, further *in vivo* validation is required before combining botanical agents with lysosomal or autophagy inhibitors can be considered a clinically applicable therapeutic strategy.

Synergistic combination regimens and chemosensitization

Given the systemic toxicities and limited survival outcomes associated with conventional cytotoxic chemotherapy in advanced CMTs, combining phytochemicals with established chemotherapeutic agents represents a potentially important strategy for enhancing antitumor activity while reducing exposure to cytotoxic drugs.

Curcumin with cyclophosphamide

Curcumin, a bioactive constituent of *Curcuma longa*, has been investigated as a chemosensitizing adjunct to cyclophosphamide, an alkylating chemotherapeutic agent associated with treatment-related adverse effects, including myelosuppression, gastrointestinal toxicity, and sterile hemorrhagic cystitis [87–89]. In CMT-U27 and CMT-U309 [67] cells, combined treatment with curcumin and cyclophosphamide produced antiproliferative synergy, altered cell cycle distribution, including G2/M- and S-phase arrest, and enhanced apoptosis [47].

Drug interaction effects were quantitatively evaluated using the Chou-Talalay median-effect method and expressed as the CI:

CI < 1.0 = synergy; CI = 1.0 = additive effect; CI > 1.0 = antagonism.

The analysis demonstrated concentration-dependent synergy between curcumin and cyclophosphamide [47]. In CMT-U27 cells, the strongest synergy occurred at one-half IC₅₀ (CI = 0.29), whereas treatment at IC₅₀ produced CI = 0.47 [47]. In CMT-U309 cells, the strongest synergy was observed at the IC₅₀ (CI = 0.42), followed by one-half IC₅₀ (CI = 0.61) [47]. These findings show that interaction magnitude depends on both cell line and treatment concentration and support further evaluation of curcumin as a potential chemosensitizing adjunct to cyclophosphamide.

Dosing regimen, schedule, and dose reduction index (DRI)

The optimized combination protocol involved concurrent exposure to curcumin and cyclophosphamide for 72 h at a fixed 1:1 molar ratio. In CMT-U27 cells, 34.20 μM curcumin combined with 5.18 mM cyclophosphamide reduced cell viability to 14.4%, whereas in CMT-U309 cells, 24.73 μM curcumin combined with 4.75 mM cyclophosphamide reduced cell viability to 35.0% [47].

The combination produced a DRI >2.0, indicating that, under the experimental conditions, the concentration of cyclophosphamide required to achieve a defined effect could be reduced by at least 50% compared with single-agent treatment [47]. However, this interaction was concentration-dependent. At lower concentrations corresponding to one-quarter and one-eighth IC₅₀, the combination lost its synergistic activity and exhibited additive or antagonistic interactions (CI ≥ 1.0) [47]. These findings indicate that the interaction between curcumin and cyclophosphamide occurs within a defined concentration range and emphasize the importance of determining whether synergistically effective exposures can be achieved and maintained *in vivo*.

Resveratrol with *V. album* extract

Resveratrol, a polyphenolic stilbenoid [90, 91], and *V. album* extract, which contains biologically active mistletoe lectins and viscotoxins [92], have been evaluated in the canine mammary tumor cell lines UNESP-CM9 and UNESP-CM60 [48]. Reported single-agent half-maximal inhibitory concentration (IC₅₀) values were 3.11 µL/100 mL and 2.993 µL/100 mL for *V. album* extract in UNESP-CM9 and UNESP-CM60 cells, respectively, and 281.6 µM and 105.5 µM for resveratrol in the corresponding cell lines [48].

Combined treatment with resveratrol and *V. album* produced greater cytotoxic and antimigratory effects than either agent alone. Under the evaluated experimental conditions, cell viability dropped to undetectable levels (0%), while scratch-wound closure fell to 2.41% compared with 39.46% in untreated controls [48]. These findings suggest complementary antitumor activity; however, without a validated interaction model, this interaction should be distinguished from formally quantified pharmacological synergy and from enhanced combination efficacy.

Mechanistic complementarity and clinical adjuvant evidence

The potential complementarity of resveratrol and *V. album* involves several intracellular and immunological pathways. *V. album* lectins have been reported to inhibit signal transducer and activator of transcription 3 (STAT3) phosphorylation and downregulate survivin [50]. Resveratrol has been associated with suppressing epithelial-mesenchymal transition-related signaling, including modulating matrix metalloproteinase (MMP)-2 and MMP-9 and increasing E-cadherin expression, along with effects on mitochondrial apoptotic pathways [90, 91].

In addition to these intracellular effects, *V. album* has demonstrated immunomodulatory activity involving cytokine responses from peripheral blood mononuclear cells (PBMCs), including interleukin-1, interferon-γ, and tumor necrosis factor-α, as well as natural killer (NK) cell-mediated cytotoxic activity [49, 50]. These systemic effects provide a mechanistic rationale for further evaluation of *V. album*-based adjunctive strategies, although mechanistic findings obtained outside spontaneous CMT models should be interpreted cautiously.

Clinical veterinary evidence provides additional support for *V. album* as an adjuvant rather than direct clinical validation of the resveratrol–*V. album* combination. In client-owned dogs with spontaneous mammary tumors, adjuvant subcutaneous *V. album* treatment was well tolerated and associated with prolonged relapse-free survival and improved quality-of-life measures, with predominantly mild injection-site reactions [68]. Thus, the clinical evidence for *V. album* should be distinguished from the preclinical evidence for its combination with resveratrol.

Critical precautionary case study: Paradoxical metastasis associated with indole-3-carbinol

Indole-3-carbinol (I3C), a phytochemical derived from cruciferous vegetables, has been investigated for antiproliferative activity in hormone-dependent human breast cancer models [93–96]. However, an *in vivo* study using an orthotopic severe combined immunodeficiency (SCID) mouse xenograft model of canine IMC demonstrated a paradoxical biological response [52]. Oral administration of I3C at 150 mg/kg/day for 3 weeks reduced tumor proliferative activity and enhanced apoptotic signaling, as indicated by decreased Ki-67 indices and increased caspase-3 expression [52]. Despite these effects on the primary tumor, I3C treatment was accompanied by increased metastatic dissemination. Dermal tumor emboli were detected in 41.7% of treated animals compared with 0% of controls, non-dermal tumor emboli in 58.3% versus 16.7%, and liver metastases in 33.3% versus 0%, respectively [52].

Steroid hormone dysregulation and metastatic progression

The paradoxical metastatic phenotype was accompanied by alterations in intratumoral steroid hormone concentrations. I3C treatment increased intratumoral estrone sulfate, estradiol, and androstenedione concentrations and was associated with elevated serum testosterone levels, while tumor embolization and liver metastasis increased despite reduced primary tumor proliferation and enhanced apoptotic signaling [52]. These findings demonstrate an association between I3C treatment, altered steroid hormone profiles, and increased metastatic dissemination in this experimental model.

The altered intratumoral hormonal environment could potentially contribute to prometastatic signaling. However, the cited experiment did not directly investigate mechanisms involving aromatase-mediated estrogen production, *CYP19A1* upregulation, or epithelial-mesenchymal transition. Therefore, these mechanisms should be regarded as hypotheses requiring experimental validation rather than established explanations for the observed metastatic phenotype [52].

Immunodeficient model limitations and implications for immune surveillance

Interpreting these findings requires considering the SCID mouse model, which lacks functional T- and B-lymphocyte-mediated adaptive immune responses. Consequently, this model does not reproduce the intact immune environment of spontaneous CMTs in immunocompetent dogs. The absence of adaptive immunity could influence tumor progression, dissemination, and the fate of circulating or intravasated tumor cells.

However, the increased metastatic burden observed after I3C administration cannot be attributed solely to the absence of immune-mediated clearance based on the reported findings. Similarly, it cannot be concluded that disseminated tumor cells would necessarily be eliminated in an immunocompetent canine host. Therefore, the SCID model represents an important limitation when extrapolating these findings to spontaneous canine disease, and validation in immunocompetent dogs with naturally occurring CMTs is required [52].

Clinical implications for veterinary oncology

Based on the available *in vivo* evidence, I3C cannot currently be recommended as monotherapy for canine IMC. The increased tumor embolization and metastatic dissemination observed despite reduced primary tumor proliferation demonstrate that antiproliferative activity alone may not predict overall disease control in aggressive mammary malignancies [52]. These findings warrant careful safety assessment and independent validation before I3C is considered for veterinary oncological use.

Future investigation of I3C or related indole compounds should first establish the mechanisms responsible for the altered steroid hormone profile and metastatic phenotype. Endocrine-targeted combinations, including aromatase inhibition, may represent a hypothesis for experimental investigation if a causal role for aromatase-dependent estrogen signaling is subsequently demonstrated. However, the currently available evidence does not support specifying aromatase inhibitors as mandatory combination partners.

Finally, the findings emphasize the limitations of evaluating therapeutic efficacy solely on the basis of primary tumor proliferation or apoptotic endpoints. Future studies should incorporate tumor growth, lymphovascular invasion, tumor embolization, distant metastasis, survival, endocrine effects, and systemic toxicity. Validation in spontaneous, immunocompetent canine mammary tumors and prospective veterinary clinical studies will be necessary before definitive conclusions regarding the clinical safety or efficacy of I3C can be established [52].

An integrated overview of the molecular targets, combination strategies, and paradoxical metastatic response associated with I3C is presented in Figure 2 [47, 48, 51, 52, 58–63, 68].

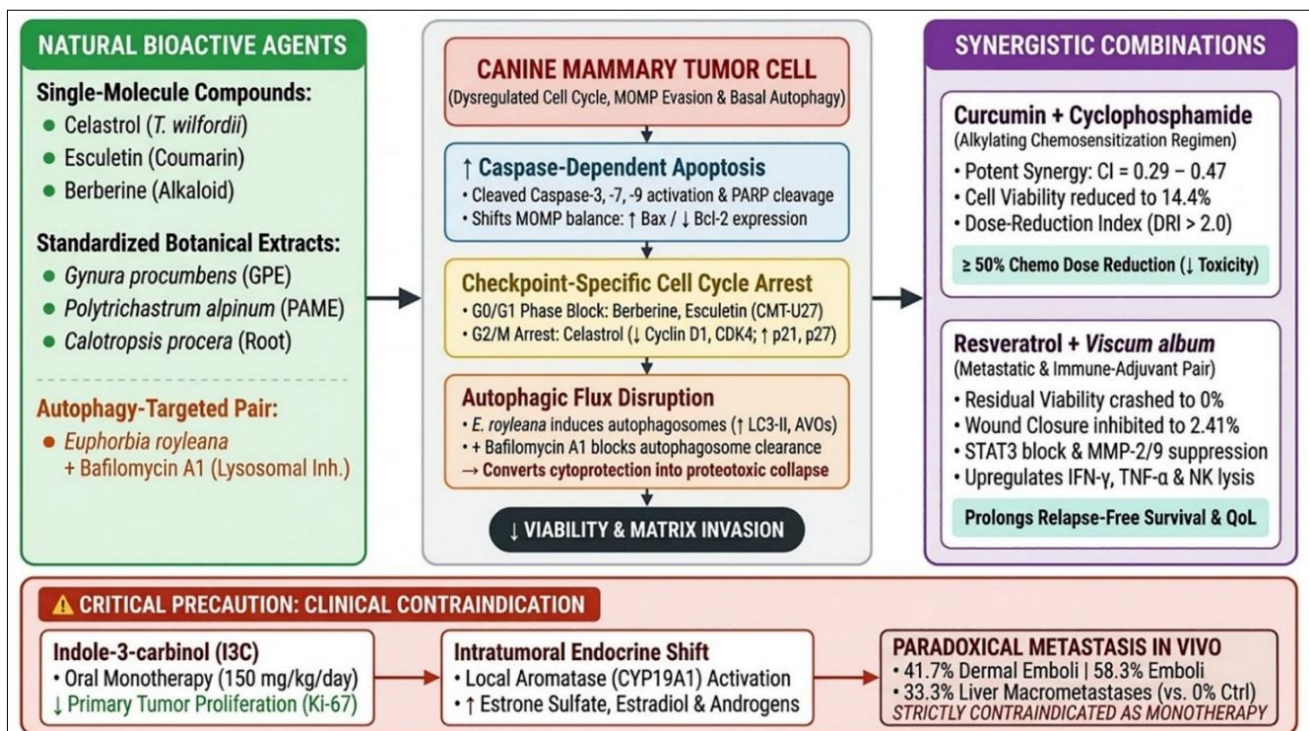


Figure 2: Anticancer mechanisms and combination strategies in canine mammary tumors [47, 48, 51, 52, 58–63, 68].

Schematic overview of molecular pathways, combination therapies, and potential safety concerns associated with phytochemicals in CMT models. Bioactive natural products modulate caspase-dependent apoptosis involving

caspase-3, caspase-7, and caspase-9 and the Bax/Bcl-2 balance [58, 60-62]; induce phase-specific cell cycle arrest at G0/G1 or G2/M through modulation of CDK4/cyclin D1 and p21/p27 [51, 58, 59, 62]; and influence autophagic responses, as demonstrated by enhanced apoptosis when *E. royleana*-induced autophagy is combined with late-stage autophagic inhibition by bafilomycin A1 [63]. Combination approaches include curcumin with cyclophosphamide (CI < 0.5 and DRI > 2.0 under specific experimental conditions) [47] and resveratrol with *V. album*, which demonstrated enhanced cytotoxic and antimigratory activity *in vitro* [48, 68]. In contrast, I3C treatment in an *in vivo* SCID mouse xenograft model was associated with altered intratumoral steroid hormone concentrations and increased tumor embolization and hepatic metastasis despite reduced primary tumor proliferation [52]. Aromatase/*CYP19A1* upregulation was not directly demonstrated and should not be considered an established mechanism.

CDK4 = cyclin-dependent kinase 4; CI = combination index; CMT = canine mammary gland tumor; DRI = dose reduction index; I3C = indole-3-carbinol; NK = natural killer; PBMC = peripheral blood mononuclear cell; SCID = severe combined immunodeficiency; STAT3 = signal transducer and activator of transcription 3. This figure was enhanced for resolution and typographic clarity using Google Gemini (Google LLC, USA).

COMPARATIVE SYNTHESIS AND TRANSLATIONAL SUMMARY OF PHYTOCHEMICAL POTENTIAL AGAINST CMTs

CMTs remain an important clinical challenge in veterinary oncology, particularly advanced malignant carcinomas and IMC, which are characterized by aggressive metastatic behavior, high mortality, and suboptimal responses to currently available systemic therapies, including chemotherapy [97, 98]. These limitations have stimulated investigation of targeted and translational therapeutic strategies. Preclinical studies conducted over the past decade indicate that bioactive phytochemicals and botanical extracts exert pleiotropic antineoplastic effects through modulation of several cancer-associated processes, including caspase activation, mitochondrial apoptotic signaling, checkpoint-specific cell cycle arrest, autophagic flux, and chemosensitization (Tables 2 and 3) [47, 48, 51, 52, 58-63, 68]. However, translation of these experimental findings into veterinary clinical oncology requires careful integration of *in vitro* potency, tumor selectivity, achievable *in vivo* exposure, safety, standardization, and canine PK characteristics.

Table 2: Qualitative antineoplastic mechanisms, study quality indicators, and translational readiness of phytochemicals evaluated in canine mammary tumor models.

Phytochemical/ botanical extract	Standardized active markers and source	Target CMT model(s)	Study quality indicators and experimental design	Primary molecular target(s) and mechanistic pathway	Key therapeutic outcomes and <i>in vitro/in vivo</i> efficacy	Critical translational barriers and limitations	Translational readiness	Reference
Celastrrol	Pentacyclic triterpenoid (>98% purity); <i>Tripterygium wilfordii</i>	CMT-7364, CIPp	<i>In vitro</i> ; n ≥ 3 biological replicates; vehicle control (DMSO); non- tumorigenic control (MDCK)	Cleaved caspase- 3/9 activation; p21/p27 induction; cyclin D1 suppression; p53-independent G2/M arrest	IC ₅₀ <1.3 μM; G2/M arrest; inhibition of migration and matrix invasion	Low aqueous solubility; absence of canine Cmax and t1/2 data; narrow systemic safety margin reported in mammals	Amber (high <i>in vitro</i> potency; requires improved delivery and <i>in vivo</i> validation)	[58]
Esculetin	6,7- Dihydroxycouma- rin (>98% purity); <i>Fraxinus rhynchophylla</i> , <i>Artemisia capillaris</i>	CMT-U27, CF41.Mg	<i>In vitro</i> ; biological replicates not reported; vehicle control (DMSO); endothelial control (CnAOECs)	Caspase-3 cleavage; transcriptional downregulation of CDK4 and cyclin D1; checkpoint- arrest	Cell line-specific G0/G1 arrest in CMT-U27 and S- phase delay in CF41.Mg; reduced viability at 0.5 mM	Low potency; submillimolar- to-millimolar concentrations required; acute endothelial cytotoxicity at 1.0 mM; narrow experimental therapeutic window	Amber (low potency; narrow safety margin)	[59]
<i>Gynura procumbens</i> leaf extract	Flavonols (quercetin, kaempferol, rutin) and chlorogenic acid; <i>G. procumbens</i>	CHMp-13a, CHMp-5b	<i>In vitro</i> ; n ≥ 3 biological replicates; negative vehicle control; normal canine control not reported	Caspase-3/7 activation; downregulation of EGFR	Dose- and time-dependent inhibition of cell proliferation and wound closure (>50% reduction at high concentrations)	Numerical IC ₅₀ in μg/mL not reported; single laboratory <i>in vitro</i> evidence; lacks canine PK data	Amber (promising extract; requires batch standardization and <i>in vivo</i> validation)	[61]

Phytochemical/ botanical extract	Standardized active markers and source	Target CMT model(s)	Study quality indicators and experimental design	Primary molecular target(s) and mechanistic pathway	Key therapeutic outcomes and <i>in</i> <i>vitro/in vivo</i> efficacy	Critical translational barriers and limitations	Translational readiness	Reference
<i>Polytrichastrum alpinum</i> extract	Benzonaphthoxa nthenones (ohioensins F and G) and moss polyphenols; <i>P. alpinum</i>	MGT316, MGT612	<i>In vitro</i> ; n ≥ 3 biological replicates; vehicle control; normal epithelial control included	Cleaved caspase-3 and PARP; Bax upregulation; Bcl-2 downregulation; G0/G1 arrest	Increased Annexin V-positive apoptotic cells; inhibition of colony formation, migration, and Matrigel invasion	Numerical IC ₅₀ not reported; evidence confined to cell culture; biomass sourcing and standardization require consideration	Amber (multitargeted activity; lacks <i>in vivo</i> evaluation)	[62]
<i>Calotropis procera</i> root extract	Cardenolides (calotropin, uscharin, calotoxin); <i>C. procera</i> root	CF41.Mg	<i>In vitro</i> ; vehicle controlled; single cell line; biological replicates and normal control not reported	Downregulation of antiapoptotic Bcl-2	Dose- dependent cytotoxicity (IC ₅₀ = 9.00 μg/mL); reduced cell viability	Bax fold change not reported; Bcl-xL and Mcl-1 not examined; potential safety concerns associated with cardiac glycosides	Amber (cytotoxic lead; systemic safety requires evaluation)	[60]
Berberine	Protoberberine isoquinoline alkaloid (>98% purity); Coptidis rhizoma	CF41.Mg	<i>In vitro</i> ; vehicle controlled; single cell line; normal control not reported	G0/G1-phase arrest; increased sub-G1 hypodiploid population	Significant proliferation suppression at 100 μM; G0/G1 population increased by 18%-22% (p < 0.005)	Numerical IC ₅₀ not reported; high effective concentration and poor oral bioavailability limit translational feasibility	Amber (high experimental concentration; requires pharmacokinetic evaluation)	[51]
<i>Euphorbia royleana</i> extract	Ingenane/ingol diterpenoids and cycloartanes; <i>E. royleana</i>	CMT cells; nude mouse xenografts	<i>In vitro</i> and <i>in vivo</i> ; biological replicates not reported; vehicle control; bafilomycin A1 used for late-stage autophagy inhibition	LC3-II accumulation; acidic vesicle formation; mutant p53 downregulation; autophagic response	Reduced tumor volume <i>in vivo</i> ; enhanced apoptosis when combined with bafilomycin A1	Extract fractions require further standardization; numerical IC ₅₀ and <i>in vivo</i> dose require clarification; combination findings require additional validation	Amber (novel mechanism; requires standardized preparations)	[63]
Curcumin + cyclophosphamide	Diferuloylmethane polyphenol (>95% purity); <i>Curcuma longa</i>	CMT-U27, CMT-U309	<i>In vitro</i> ; biological replicates not reported; vehicle control; concurrent combination testing; Chou- Talalay modeling	Mitochondrial apoptotic modulation; G2/M- synergistic and S-phase arrest; chemosensitization	CI = 0.29-0.47 under strongly synergistic conditions; DRI >2.0 under specified experimental conditions	Poor systemic exposure of free curcumin; interaction becomes additive or antagonistic at lower concentrations	Green (strong preclinical combination potential; requires <i>in vivo</i> PK and efficacy validation)	[47]
Resveratrol + <i>Viscum album</i> extract	Stilbenoid polyphenol combined with mistletoe lectins and viscotoxins	UNESP-CM9, UNESP- CM60; separate clinical evidence for <i>V. album</i> in client- owned dogs	<i>In vitro</i> combination assays; separate veterinary clinical adjuvant evidence for <i>V. album</i>	STAT3-associated signaling, MMP-related pathways, E-cadherin, and immunomodulatory mechanisms reported across supporting evidence	Combination reduced cell viability and wound closure <i>in vitro</i> ; <i>V. album</i> separately associated with clinical adjuvant benefits in dogs	Rapid phase II metabolism of free resveratrol; clinical evidence does not directly validate the resveratrol- <i>V. album</i> combination	Green (promising combination; partial clinical support derives from <i>V. album</i> independently)	[48, 68]
Indole-3-carbinol	Glucobrassicin- derived indole; cruciferous vegetables	Orthotopic SCID mouse xenograft of canine IMC	<i>In vivo</i> ; controlled xenograft experiment; n = 12 mice/group; necropsy and histopathological evaluation	Reduced Ki-67 and increased caspase- 3; altered intratumoral steroid hormone profiles	Reduced primary tumor proliferation but increased tumor embolization and hepatic metastasis	Dermal emboli, 41.7%; liver metastasis, 33.3%; immunodeficient model; mechanism underlying metastatic increase remains unresolved	Red/Caution (paradoxical metastatic findings require further safety investigation)	[52]

Data on experimental quality indicators, biological replicates, and controls were extracted from the primary studies; unavailable parameters are designated as "Not reported." Translational readiness score: Green = comparatively higher translational potential based on validated combination effects and/or supporting canine clinical evidence; Amber = promising preclinical activity constrained by poor bioavailability, high effective concentrations, narrow safety margins, insufficient standardization, or lack of canine *in vivo* evidence; Red = critical safety concern based on paradoxical adverse *in vivo* findings requiring further investigation before clinical translation. CDK4 = cyclin-dependent kinase 4; CI = combination index; CMT = canine mammary gland tumor; CnAOECs = canine aortic endothelial cells; DMSO = dimethyl sulfoxide; DRI = dose reduction index; EGFR = epidermal growth factor receptor; IC₅₀ = half-maximal inhibitory concentration; IMC = inflammatory mammary carcinoma; MDCK = Madin-Darby canine kidney; MMP = matrix metalloproteinase; PARP = poly(ADP-ribose) polymerase; PK = pharmacokinetics; SCID = severe combined immunodeficiency; STAT3 = signal transducer and activator of transcription 3.

COMPARATIVE SYNTHESIS AND TRANSLATIONAL ASSESSMENT

Caspase-dependent apoptotic activation

Celastrol: Celastrol demonstrated the highest *in vitro* apoptotic potency among the evaluated single-molecule phytochemicals, with IC₅₀ values <1.3 μ M in CMT-7364 and CIPp cells and activation of caspase-3 and caspase-9 [58].

Translational qualifier: Although celastrol demonstrated a favorable selectivity profile relative to non-tumorigenic MDCK cells and potent caspase-dependent proapoptotic activity in CMT cell lines, its translation into veterinary oncology remains uncertain. Poor aqueous solubility, limited bioavailability, and systemic toxicity may limit effective therapeutic exposure *in vivo*. Advanced delivery approaches, including liposomal and nanoparticle-based formulations, have therefore been proposed to improve systemic exposure and potentially reduce off-target toxicity [58, 99].

Esculetin: Esculetin induced caspase-3-mediated apoptosis but required substantially higher, submillimolar concentrations (approximately 0.5 mM) to produce significant antitumor effects [59].

Translational qualifier: Esculetin demonstrated a relatively narrow experimental therapeutic window because cytotoxicity toward CnAOECs became evident at approximately 1.0 mM. Consequently, formulation optimization or targeted-delivery approaches may be required before its translational potential can be adequately evaluated [59].

Standardized *G. procumbens* leaf extract: Standardized *G. procumbens* leaf extract induced caspase-3/7-mediated apoptosis together with EGFR downregulation in CHMp-13a and CHMp-5b cells ($p < 0.05$) [61].

Translational qualifier: Evidence remains restricted to *in vitro* models and requires independent confirmation. Chemical standardization of major bioactive markers, including quercetin and kaempferol, together with batch-to-batch compositional characterization and independent biological validation, should precede further veterinary translational evaluation [61, 74].

***P. alpinum* methanol extract:** *P. alpinum* methanol extract induced apoptosis and cell cycle arrest in MGT316 and MGT612 cells while exhibiting minimal cytotoxicity toward the non-tumorigenic control cells evaluated in the study [62].

Translational qualifier: Current evidence is limited to preclinical cell models. Independent replication, chemical standardization, and subsequent *in vivo* pharmacokinetic, safety, and efficacy studies are required before its translational potential can be established [62, 75].

Modulation of the mitochondrial Bcl-2/Bax apoptotic rheostat

***C. procera* root extract:** *C. procera* root extract, containing bioactive cardenolides such as calotropin and uscharin, demonstrated significant antiproliferative activity against CF41.Mg cells and downregulated Bcl-2 expression (IC₅₀ = 9.0 μ g/mL, $p < 0.05$) [60, 79].

Translational qualifier: Although *C. procera* root extract demonstrated Bcl-2 downregulation in CF41.Mg cells, the available evidence is insufficient to establish comprehensive modulation of the mitochondrial Bcl-2/Bax rheostat. Bax expression and the Bax/Bcl-2 ratio were not quantified, and other antiapoptotic proteins, including Bcl-xL and Mcl-1, were not evaluated. These omissions limit assessment of mitochondrial apoptotic commitment and potential compensatory resistance mechanisms. Furthermore, because the extract contains cardenolides with cardiac glycoside-like pharmacological activity, dedicated *in vivo* cardiac safety evaluation in dogs is required before clinical translation [60, 79].

Checkpoint-specific cell cycle arrest

Berberine: Berberine induced G0/G1-phase arrest in CF41.Mg cells, increasing the G0/G1 population by approximately 18%-22% at 100 μ M ($p < 0.005$) [51].

Translational qualifier: Although berberine produced significant G0/G1-phase arrest at 100 μ M, the clinical relevance of this concentration remains uncertain. Poor oral bioavailability associated with intestinal P-glycoprotein-mediated efflux and extensive first-pass metabolism may restrict systemic exposure to the parent compound *in vivo* [51, 81]. Canine-specific pharmacokinetic studies are therefore required to determine whether biologically relevant exposure can be achieved safely.

Celastrol: Celastrol induced pronounced G2/M-phase arrest in CMT-7364 and CIPp cells. The G2/M population increased to 32.7% in CMT-7364 cells and approximately 34.4%

in CIPp cells at the highest evaluated concentration ($p < 0.01$), accompanied by increased p21 and p27 expression and reduced cyclin D1 expression [58]. These findings support activation of cell cycle regulatory mechanisms that restrict G2/M progression.

Translational qualifier: The available study did not directly establish the dependence of this response on *TP53* signaling. Therefore, although celastrol demonstrated checkpoint-specific activity, mechanistic conclusions regarding *TP53*-independent regulation require direct experimental validation [58].

Esculetin: Esculetin produced divergent cell cycle responses, inducing G0/G1-phase arrest in CMT-U27 cells, with the G0/G1 population increasing from 54.2% to 68.2%, whereas CF41.Mg cells exhibited an S-phase delay, with the S-phase population increasing from 24.5% to 36.5% [59].

Translational qualifier: These divergent responses demonstrate cell line-specific sensitivity but do not establish that molecular lineage itself is responsible for the difference. Additional molecular characterization is needed to determine whether CDK signaling, molecular subtype, or other regulatory mechanisms explain the observed response heterogeneity.

Autophagic flux modulation as an antineoplastic strategy

***E. royleana* extract:** *E. royleana* extract induced an autophagic response in CMT cells characterized by LC3-II accumulation and increased acidic vesicular organelle formation [63].

Translational qualifier: The treatment-associated autophagic response appeared to exert a predominantly cytoprotective function because pharmacological inhibition of late-stage autophagy with bafilomycin A1 significantly enhanced apoptosis [63]. These findings suggest that autophagic flux may limit maximal extract-mediated cytotoxicity under the experimental conditions. However, translational development requires chemical standardization of the extract, independent confirmation of the mechanism, and validation in clinically relevant *in vivo* models, including immunocompetent canine disease.

Chemotherapeutic synergy and co-therapy regimens

Curcumin with cyclophosphamide: Curcumin combined with cyclophosphamide demonstrated strong, concentration-dependent synergy in CMT-U27 and CMT-U309 cells. Under strongly synergistic conditions, CI values reached 0.29 in CMT-U27 cells and 0.42 in CMT-U309 cells, and cell viability dropped to 14.4% under the reported CMT-U27 combination regimen. DRI values >2.0 indicated substantial experimental dose reduction at defined effect levels [47].

Translational qualifier: The interaction was strongly concentration-dependent, with lower fractional concentrations producing additive or antagonistic responses. Furthermore, the *in vitro* study did not establish pharmacokinetic feasibility in dogs. Canine-specific studies are therefore required to determine whether curcumin and cyclophosphamide exposures associated with synergistic activity can be achieved simultaneously and safely *in vivo* [47].

Resveratrol with *V. album* extract: Resveratrol combined with *V. album* extract produced marked cytotoxic and antimigratory effects in UNESP-CM9 and UNESP-CM60 cells, including reduction of cell viability to 0% under the evaluated conditions and pronounced inhibition of scratch-wound closure [48]. Mechanistic evidence indicates that *V. album* constituents can modulate STAT3-associated signaling, whereas resveratrol affects multiple pathways associated with tumor cell survival and metastatic behavior [48, 100].

Translational qualifier: Clinical veterinary evidence for *V. album* should be distinguished from the preclinical evidence for the resveratrol-*V. album* combination. Subcutaneous *V. album* has demonstrated an acceptable safety profile and potential clinical benefits in dogs, whereas oral resveratrol undergoes extensive phase II metabolism through glucuronidation and sulfation, limiting systemic exposure to the parent compound [56, 68, 101]. Consequently, formulation optimization and canine pharmacokinetic evaluation are required before the translational feasibility of the combination can be established.

Critical clinical precaution: Paradoxical prometastatic risk associated with indole-3-carbinol

Indole-3-carbinol: In an orthotopic SCID mouse xenograft model of canine IMC, oral I3C at 150 mg/kg/day reduced primary tumor proliferative activity, as demonstrated by decreased Ki-67 expression, but was accompanied by increased metastatic dissemination [52]. Dermal tumor emboli occurred in 41.7% of treated animals compared with 0% of controls, non-dermal emboli occurred in 58.3% versus 16.7%, and liver metastases occurred in 33.3% versus 0%, respectively [52].

Translational qualifier: The paradoxical metastatic phenotype coincided with alterations in intratumoral steroid hormone profiles, including increased estrone sulfate and estradiol concentrations [52]. These findings suggest a potential association between altered steroid hormone signaling and metastatic progression; however, the underlying causal mechanism was not established. In particular, aromatase/*CYP19A1* upregulation and epithelial-

mesenchymal transition were not directly demonstrated and should not be presented as established mechanisms. The increased metastatic dissemination despite suppression of primary tumor proliferation raises an important safety concern regarding I3C as a standalone intervention for advanced or inflammatory mammary tumors. Nevertheless, because these findings were generated in an immunodeficient xenograft model, validation in spontaneous CMTs in immunocompetent dogs is required before definitive clinical conclusions can be drawn [52]. A comprehensive quantitative synthesis of effect measures, IC₅₀ values, CI values, and reported experimental reproducibility indicators across the evaluated studies is presented in Table 3 [47, 48, 51, 52, 58–63, 68].

Table 3: Quantitative potency metrics, apoptotic endpoints, checkpoint-arrest kinetics, and reproducibility indicators for phytochemicals evaluated in CMT models.

Phytochemical/treatment	Target CMT model	Quantitative potency metrics/effective concentrations	Quantitative effects and mechanistic endpoints	Study conditions and reproducibility indicators	Reference
Celastrrol	CMT-7364; CIPp	CMT-7364: IC ₅₀ = 1.236 μ M (24 h) and 1.007 μ M (48 h); CIPp: IC ₅₀ = 1.156 μ M (24 h) and 0.968 μ M (48 h)	Viability approached 0% at 1.6 μ M ($p < 0.01$). CMT-7364 G2/M population increased from 5.6% to 20.3% (0.3 μ M) and 32.7% (0.9 μ M); CIPp increased from 12.0% to 21.9% and 34.4%, respectively ($p < 0.01$)	Single laboratory; two carcinoma cell lines; molecular validation by quantitative real-time polymerase chain reaction and Western blotting; MDCK control included	[58]
Esculetin	CMT-U27; CF41.Mg	Effective concentrations: 0.5 and 1.0 mM; numerical IC ₅₀ not reported	CMT-U27: viability reduced by 40% at 24 h and 80% at 48 h ($p < 0.05$); G0/G1 population increased from 54.2% to 68.2%. CF41.Mg: viability reduced by 20% and 35% at 24 and 48 h, respectively ($p < 0.05$); S-phase population increased from 24.5% to 36.5% at 1.0 mM ($p < 0.05$)	Single laboratory; two cell lines; Western blotting for CDK4/cyclin D1; CnAOEC control included, with significant cytotoxicity at 1.0 mM ($p < 0.01$)	[59]
<i>Gynura procumbens</i> leaf extract	CHMp-13a; CHMp-5b	High-concentration ethanolic extract; numerical IC ₅₀ in μ g/mL not reported	Viability and migration reduced by >50% ($p < 0.05$); caspase-3/7 activity significantly increased ($p < 0.05$); quantitative EGFR fold reduction not reported	Single laboratory; two CMT cell lines; scratch-wound assay; normal canine control not reported	[61]
<i>Polytrichastrum alpinum</i> extract	MGT316; MGT612	Effective concentration range: 12.5–100 μ g/mL; numerical IC ₅₀ not reported	Annexin V-positive apoptotic fractions significantly increased ($p < 0.05$); G0/G1 arrest; increased cleaved caspase-3 and PARP; quantitative Bax/Bcl-2 fold change not reported	Single laboratory; two carcinoma cell lines; Matrigel transwell invasion assay; normal epithelial control included with negligible cytotoxicity	[62]
<i>Calotropis procera</i> root extract	CF41.Mg	IC ₅₀ = 9.00 μ g/mL at 24 h	Significant Bcl-2 downregulation ($p < 0.05$); quantitative Bax/Bcl-2 ratio not reported; cell cycle redistribution not reported	Single laboratory; single cell line; Western blot analysis; orthogonal molecular validation not reported	[60]
Berberine	CF41.Mg	Effective concentration: 100 μ M at 24 h; numerical IC ₅₀ not reported	Significant reduction in viability at 100 μ M ($p < 0.005$); G0/G1 population increased by 18%–22% compared with controls ($p < 0.005$); sub-G1 population significantly increased ($p < 0.005$)	Single laboratory; single cell line; flow cytometric cell cycle analysis; normal canine control not reported	[51]
<i>Euphorbia royleana</i> extract	CMT cells; nude mouse xenografts	Hexane and ethanol fractions; numerical IC ₅₀ and <i>in vivo</i> dose in mg/kg not reported	Increased LC3-II and decreased mutant p53; increased acidic vesicular organelles; significant reduction in tumor volume <i>in vivo</i> ($p < 0.05$); 10 nM bafilomycin A1 enhanced apoptosis	Single laboratory; evaluated <i>in vitro</i> and in nude mouse xenografts; autophagic response assessed using lysosomal inhibitor co-treatment	[63]
Curcumin + cyclophosphamide	CMT-U27; CMT-U309	CMT-U27: 34.20 μ M curcumin + 5.18 mM cyclophosphamide; CMT-U309: 24.73 μ M curcumin + 4.75 mM cyclophosphamide	CMT-U27: CI = 0.29 at one-half IC ₅₀ and 0.47 at IC ₅₀ ; CMT-U309: CI = 0.42 at IC ₅₀ and 0.61 at one-half IC ₅₀ . DRI >2.0; viability reduced to 14.4% and 35.0%, respectively	Two CMT cell lines; concurrent 72-h exposure; Chou-Talalay modeling; additive or antagonistic interactions observed at one-quarter and one-eighth IC ₅₀ (CI ≥ 1.0)	[47]

Phytochemical/ treatment	Target CMT model	Quantitative potency metrics/effective concentrations	Quantitative effects and mechanistic endpoints	Study conditions and reproducibility indicators	Reference
Resveratrol + <i>Viscum album</i> extract	UNESP-CM9; UNESP-CM60	Reported single-agent values: <i>V. album</i> , 3.11 µL/100 mL (UNESP-CM9) and 2.993 µL/100 mL (UNESP-CM60); resveratrol, 281.6 µM and 105.5 µM, respectively	Combined treatment reduced viability to 0% under the evaluated conditions; UNESP-CM60 wound closure reduced to 2.41% versus 39.46% in controls ($p < 0.05$)	Two CMT cell lines; <i>in vitro</i> viability and scratch-wound assays; separate canine clinical adjuvant evidence available for <i>V. album</i>	[48, 68]
Indole-3-carbinol	Orthotopic SCID mouse xenograft of canine IMC	Oral dose: 150 mg/kg/day for 21 days	Reduced Ki-67 and increased caspase-3; dermal tumor emboli, 41.7% versus 0% in controls; non- dermal emboli, 58.3% versus 16.7%; hepatic metastasis, 33.3% versus 0%	<i>In vivo</i> orthotopic xenograft (n = 12/group); hormone profiling demonstrated increased intratumoral estrone sulfate, estradiol, and androstenedione	[52]

All quantitative metrics, including IC₅₀ values, CI values, cell cycle population shifts, and apoptotic endpoints, were extracted from the included primary studies. Parameters that were unavailable or not measured are explicitly designated as “Not reported” to improve transparency and prevent inference of unmeasured outcomes.

CDK4 = cyclin-dependent kinase 4; CI = combination index; CMT = canine mammary gland tumor; CnAOEC = canine aortic endothelial cell; DRI = dose reduction index; EGFR = epidermal growth factor receptor; IC₅₀ = half-maximal inhibitory concentration; IMC = inflammatory mammary carcinoma; MDCK = Madin-Darby canine kidney; PARP = poly(ADP-ribose) polymerase; SCID = severe combined immunodeficiency.

TRANSLATIONAL CHALLENGES, CLINICAL EVIDENCE, AND FUTURE DIRECTIONS

Despite substantial preclinical evidence demonstrating the antineoplastic activity of phytochemicals in CMT cell lines, major barriers limit their progression from experimental models to evidence-based veterinary oncology [33]. Most available evidence remains derived from isolated *in vitro* monocultures that do not reproduce the complex tumor microenvironment, immune surveillance, drug metabolism, and PK characteristics of canine patients [33, 34]. Future research should therefore extend beyond repetitive cytotoxicity screening of poorly characterized botanical preparations and prioritize pharmacokinetic characterization, formulation standardization, target-tissue exposure, safety assessment, and biomarker-driven clinical evaluation in dogs with spontaneous CMTs [33, 102].

Existing clinical and real-world evidence in spontaneous CMTs

Direct clinical evidence evaluating phytochemicals in client-owned dogs with spontaneous CMTs remains limited. Standardized aqueous extracts of *V. album* represent one of the more clinically evaluated botanical interventions in companion animal oncology [49, 68]. In dogs with naturally occurring mammary carcinoma, adjuvant administration of *V. album* following surgery has been associated with prolonged disease-free intervals and reduced recurrence [68]. Reported adverse events were generally mild and transient and consisted predominantly of local injection-site reactions, while potential improvements in patient well-being and quality-of-life have also been reported [68].

In addition, Withers *et al.* [103] evaluated liposome-encapsulated curcumin in client-owned dogs with naturally occurring cancers and demonstrated the feasibility of systemic administration. Although these findings were not specific to CMTs, they provide clinically relevant evidence supporting further investigation of curcumin-based formulations in companion animal oncology.

Complementary and botanical products are also used by owners of companion animals with cancer [104, 105]. However, the extent of their use specifically among dogs with mammary tumors and the frequency with which owners disclose such use to veterinary oncologists require careful verification before precise prevalence estimates are stated. Unsupervised administration nevertheless raises clinically relevant concerns regarding herb-drug interactions, variability in product composition and quality, and adverse effects, emphasizing the need for evidence-based veterinary guidance [33, 104].

Canine-specific bioavailability and pharmacokinetic barriers

Unfavorable PK characteristics represent a major barrier to translating several phytochemical candidates from *in vitro* experiments to canine patients [53, 56, 101]. Extrapolation from rodent studies alone is insufficient because clinically relevant development requires direct characterization of systemic exposure, metabolism, clearance, tissue distribution, and safety in dogs.

Curcumin: Free oral curcumin demonstrates poor systemic exposure in dogs, with reported peak plasma concentrations in the low nanogram-per-milliliter range (<25 ng/mL), substantially below the micromolar concentrations used for apoptosis induction and chemosensitization *in vitro* [47, 53, 91]. Intravenous administration of liposomal

curcumin at 10 mg/kg in research beagles produced transient micromolar plasma exposure; however, systemic elimination was rapid, with a reported plasma half-life of approximately 24-42 min and detectable formation of tetrahydrocurcumin, indicating extensive metabolic conversion *in vivo* [55]. These findings support formulation-based approaches but also demonstrate that improved delivery does not eliminate the need to characterize exposure duration and target-tissue concentrations.

Resveratrol: Free trans-resveratrol undergoes extensive presystemic metabolism following oral administration in dogs [56]. Intestinal and hepatic phase II metabolism rapidly converts the parent compound into glucuronide and sulfate conjugates, substantially reducing systemic exposure to unchanged resveratrol [56, 101]. This metabolic profile creates a major discrepancy between the concentrations used in CMT cell experiments and the exposure likely to be achieved after conventional oral administration.

Berberine: Berberine produced significant biological effects in CF41.Mg cells at 100 μ M [51]. However, oral berberine has poor systemic bioavailability because of limited intestinal absorption, transporter-mediated efflux, and extensive first-pass metabolism [51, 81]. Consequently, whether systemic or intratumoral concentrations approaching those used *in vitro* can be achieved safely in dogs remains uncertain and requires direct canine pharmacokinetic investigation.

Collectively, these limitations indicate that additional screening based solely on *in vitro* cytotoxicity provides limited translational information when systemic exposure and target-tissue concentrations remain unknown. Therefore, priority should be given to pharmacokinetic characterization, tumor biodistribution, formulation development, and safety assessment of the most promising candidates before advancing additional efficacy claims [33, 53, 102].

Regulatory frameworks and compliance pathways for veterinary botanicals

Regulatory requirements represent an additional challenge to integrating phytochemical therapeutics into evidence-based veterinary medicine, and requirements differ substantially among jurisdictions [64, 106, 107].

In the United States, botanical products intended to diagnose, cure, mitigate, treat, or prevent disease in animals may fall within the regulatory framework governing animal drugs. Consequently, products developed specifically for treatment of CMTs would require an appropriate regulatory development pathway addressing product quality, target-animal safety, and therapeutic efficacy [64]. Botanical products marketed without therapeutic claims are subject to different regulatory considerations and should not be interpreted as clinically validated anticancer therapeutics.

Within the European Union, veterinary medicinal products are regulated under Regulation (EU) 2019/6 [106]. Translation of botanical candidates into authorized veterinary therapeutics therefore requires appropriate documentation of pharmaceutical quality, safety, and efficacy. The precise regulatory pathway depends on the nature, intended use, formulation, and regulatory classification of the product and should be evaluated individually rather than extrapolated from pathways applicable to human traditional herbal medicines.

From a manufacturing perspective, botanical therapeutic candidates require rigorous CMC characterization. Relevant quality control measures include chromatographic fingerprinting using high-performance liquid chromatography or liquid chromatography-tandem mass spectrometry, quantitative specification of active or marker compounds, batch-to-batch consistency, and appropriate testing for contaminants such as heavy metals, pesticide residues, microorganisms, and mycotoxins [102, 108]. Such standardization is essential because reproducible pharmacological activity cannot be established when extract composition varies substantially among batches.

Advanced formulations: Scalability, stability, and feasibility

Advanced delivery systems, including solid lipid nanoparticles, liposomes, and phytosomal formulations, have been investigated to improve the solubility, stability, systemic exposure, or tissue delivery of poorly bioavailable phytochemicals [45, 109]. However, formulation development introduces additional translational challenges. Conventional aqueous liposomes may undergo phospholipid oxidation, drug leakage, aggregation, and other physicochemical changes during storage [109]. Alternative lipid-based systems and lyophilized formulations may improve stability, but particle size, drug loading, release kinetics, storage stability, and biological performance must be demonstrated for each formulation rather than assumed from the delivery platform itself [53, 109].

Manufacturing scale-up also requires reproducible control of particle characteristics, sterility, composition, and batch uniformity under appropriate manufacturing standards [102, 108, 109]. Techniques that perform adequately at laboratory scale may require substantial optimization during transition to larger-scale manu-

facturing. Therefore, formulation efficacy, manufacturability, stability, and quality control should be developed concurrently rather than treating nanoformulation as an automatic solution to poor bioavailability.

Economic feasibility and owner adherence are additional considerations in companion animal oncology. Advanced formulations may increase treatment costs and administration complexity. Consequently, clinical adoption will ultimately depend on demonstrating that improved pharmacological exposure produces meaningful benefits in tumor control, quality-of-life, or survival that justify the additional treatment burden and expense.

Biomarker-driven neoadjuvant window-of-opportunity trials

Rather than relying exclusively on broad recommendations for future clinical trials, translational research could employ biomarker-driven neoadjuvant window-of-opportunity designs in client-owned dogs with spontaneous CMTs [33, 110]. Such studies could administer a candidate phytochemical formulation, alone or in combination with an established treatment, during the interval between diagnostic evaluation and definitive surgery without unnecessarily delaying standard surgical management. Paired pretreatment biopsy specimens and post-treatment surgical specimens obtained from the same patient would permit within-subject assessment of pharmacodynamic responses while reducing variability associated with the genetic and biological heterogeneity of outbred canine populations [18, 20, 27, 31, 35, 38]. Relevant endpoints could include changes in Ki-67 proliferation indices, cleaved caspase-3, microvessel density, and other pathway-specific biomarkers selected according to the candidate's proposed mechanism. Such early-phase studies should incorporate pharmacokinetic sampling, tumor tissue drug measurements, safety monitoring, and prespecified pharmacodynamic endpoints. Dose, treatment duration, sample size, statistical power, and combination regimen should be established from preceding canine pharmacokinetic and safety data rather than predetermined solely from *in vitro* efficacy. This sequential approach would provide stronger evidence for deciding whether a candidate warrants subsequent randomized trials evaluating recurrence, progression, and survival.

Comparative oncology and One Health translational value

Spontaneous canine mammary carcinomas offer a potentially valuable comparative model for human breast cancer because naturally occurring tumors develop in immunocompetent hosts and share selected histopathological, molecular, and biological characteristics with human mammary malignancies [14, 20, 25, 28]. Comparative features include histological grading systems, molecular phenotypes with similarities to luminal and triple-negative disease, and alterations involving oncogenic pathways and genes such as *TP53* and *PIK3CA* [21, 27, 30]. Companion dogs also share aspects of the human environment and may experience overlapping environmental exposures [5, 29]. Accordingly, carefully designed studies of pharmacokinetically optimized phytochemicals and rational combination regimens in dogs with spontaneous CMTs could help bridge the translational gap between reductionist cell culture systems, experimental rodent xenografts, and naturally occurring malignancies. This comparative approach may generate clinically relevant information for veterinary patients while contributing to broader One Health and comparative oncology research [29, 33, 34, 47] (Table 4) [29, 37, 41, 47, 48, 53, 55, 64, 65, 68, 89, 102, 103, 106, 111–113]. Additionally, a methodological limitation of this review is that the literature screening, eligibility assessment, and data extraction were performed by a single investigator without independent duplicate verification.

Table 4: Prioritized strategic research roadmap for the translation of phytochemicals into canine mammary oncology.

Priority and evidence strength	Focus area	Core research question and proposed study design	Sample size and methodological considerations	Strategic translational and One Health objectives	Indicative timeline	Translational feasibility	Reference
Priority 1 (strong evidence for a major PK barrier)	PK and safety of lipid-based formulations	Determine the maximum tolerated dose, dose-limiting toxicities, and canine PK parameters (C _{max} , AUC, and t _{1/2}) of nanoencapsulated versus free curcumin. Design: Prospective comparative pharmacokinetic and tolerability study in dogs	Cohort size should be determined using formulation-specific variance and expected exposure differences from preliminary data. Serial plasma sampling with validated LC-MS/MS should quantify the parent compound and major metabolites	Establish whether formulation improves systemic exposure sufficiently to justify efficacy testing; characterize safety and metabolic disposition before trials in dogs with spontaneous CMTs	12–18 months	Green (delivery platforms are available, but canine dose and exposure require validation)	[53, 55, 103]

Priority and evidence strength	Focus area	Core research question and proposed study design	Sample size and methodological considerations	Strategic translational and One Health objectives	Indicative timeline	Translational feasibility	Reference
Priority 2 (strong preclinical combination evidence)	Biomarker-driven neoadjuvant efficacy trial	Determine whether nano-curcumin combined with low-dose cyclophosphamide safely enhances intratumoral apoptosis and suppresses proliferation in spontaneous CMTs. Design: Prospective, randomized, controlled window-of-opportunity study conducted before definitive surgery	Sample size should be determined prospectively from the primary biomarker endpoint and variance estimates obtained from pilot data. Paired pretreatment and surgical specimens should assess Ki-67, cleaved caspase-3, and other prespecified pharmacodynamic biomarkers. PK and safety assessments should be incorporated	Determine whether the <i>in vitro</i> chemosensitizing interaction translates into measurable intratumoral biological activity without delaying standard surgical management	24–36 months	Green (strong preclinical rationale, but canine PK and dose-finding should precede efficacy testing)	[47, 65, 89, 111]
Priority 3 (moderate evidence; clinical evidence primarily supports <i>Viscum album</i> independently)	Antimetastatic and immune-adjuvant clinical evaluation	Determine whether a standardized <i>V. album</i> -based adjuvant strategy, with or without an optimized resveratrol formulation, improves disease control in dogs with aggressive spontaneous mammary carcinoma. Design: Prospective, multicenter, randomized controlled trial following appropriate preliminary safety and PK studies	Sample size should be based on a prespecified primary clinical endpoint and expected event rate. Potential endpoints include disease-free interval, progression-free survival, overall survival, metastatic progression, adverse events, and selected immune biomarkers	Establish whether preclinical combination activity and the separate clinical signal for <i>V. album</i> translate into clinically meaningful benefit; provide comparative oncology evidence relevant to aggressive mammary malignancies	36–48 months	Amber (potentially high clinical value but dependent on multicenter recruitment and preceding formulation studies)	[29, 37, 41, 48, 65, 68]
Priority 4 (moderate evidence; standardization is a prerequisite for development)	CMC standardization of botanical extracts	Determine whether analytical fingerprinting, quantitative marker analysis, and botanical authentication can establish reproducible composition and stability of multiconstituent extracts, including <i>Euphorbia royleana</i> and <i>Gynura procumbens</i> . Design: Multibatch analytical characterization incorporating different harvests and stability assessment	Multiple geographically and seasonally distinct batches should be analyzed with appropriate analytical replication. HPLC and/or LC-MS/MS should quantify predefined marker compounds, while validated botanical authentication and stability methods should be incorporated	Minimize batch-to-batch chemical heterogeneity; establish identity, purity, potency, and stability specifications required for subsequent pharmacological, toxicological, and regulatory development	12–24 months; may proceed in parallel with other preclinical development	Amber (technically feasible but requires validated analytical methods, standardized sourcing, and appropriate manufacturing infrastructure)	[64, 102, 106, 112, 113]

Research priorities are ranked according to the strength of the available preclinical and clinical evidence, pharmacological feasibility, major translational barriers, and potential value for spontaneous canine disease and comparative oncology. The proposed timelines are indicative rather than validated development schedules. Formal sample size calculations should be performed prospectively for each study using a prespecified primary endpoint, expected effect magnitude, variance, anticipated attrition, and clinically acceptable type I and type II error rates. Translational feasibility categories are qualitative: Green indicates comparatively high feasibility based on an established experimental rationale or available delivery technology, whereas Amber indicates moderate feasibility requiring additional pharmacological, manufacturing, multicenter, or regulatory development.

AUC = area under the plasma concentration-time curve; CMC = chemistry, manufacturing, and controls; CMT = canine mammary gland tumor; Cmax = maximum plasma concentration; HPLC = high-performance liquid chromatography; LC-MS/MS = liquid chromatography-tandem mass spectrometry; PK = pharmacokinetics; t_{1/2} = elimination half-life.

CONCLUSION

Preclinical investigations published from January 2014 to August 2026 demonstrate that selected bioactive phytochemicals and standardized botanical extracts can modulate multiple cancer-associated pathways in CMT models, including caspase-dependent apoptosis, mitochondrial Bcl-2/Bax signaling, checkpoint-specific cell cycle arrest, autophagic responses, and chemosensitization [33, 34]. However, the overall evidence remains predominantly preclinical. Most mechanistic findings are derived from isolated *in vitro* cell line models, while *in vivo* evidence is limited largely to non-canine murine models, and prospective clinical trials specifically evaluating

these interventions in dogs with spontaneous CMTs remain lacking [33, 58].

Important methodological limitations further restrict translation. The small number of eligible experimental studies and the predominance of favorable findings suggest a potential risk of publication bias, although the available evidence cannot formally determine its magnitude. In addition, substantial disparities exist between concentrations demonstrating activity *in vitro* and exposures likely to be achievable *in vivo*. Poor aqueous solubility, extensive metabolism, limited oral bioavailability, and rapid systemic clearance represent major pharmacological barriers for several candidate compounds [53, 56]. Consequently, *in vitro* potency alone should not be interpreted as evidence of clinical therapeutic feasibility.

Among the evaluated approaches, combination strategies provide a strong rationale for further translational investigation. Curcumin combined with cyclophosphamide demonstrated concentration-dependent synergy, with CI values <0.5 under selected experimental conditions and DRI values >2.0 [47]. These findings support further investigation of curcumin as a chemosensitizing adjunct but do not establish that equivalent chemotherapy dose reductions or toxicity mitigation will occur clinically in dogs. Similarly, resveratrol combined with *V. album* showed enhanced cytotoxic and antimigratory activity *in vitro*, and separate veterinary clinical evidence for *V. album* supports investigating standardized botanical adjuvant strategies [48, 68]. The clinical evidence for *V. album*, however, should not be interpreted as direct clinical validation of the resveratrol–*V. album* combination.

Celastrol demonstrated the greatest *in vitro* potency among the evaluated single-molecule candidates, with IC₅₀ values <1.3 µM and pronounced G2/M-phase arrest [58]. Nevertheless, its poor solubility, limited bioavailability, and potential systemic toxicity necessitate formulation development, pharmacokinetic characterization, and safety assessment before clinical efficacy studies. Multicomponent extracts, including *E. royleana*, *G. procumbens*, and *P. alpinum*, also demonstrated promising multitarget activity but require rigorous chemical characterization, batch standardization, and CMC development before reproducible pharmacological evaluation [61–63, 102].

A major strength of this review is integrating mechanistic efficacy with potency, tumor selectivity, pharmacological feasibility, safety, study reproducibility, and translational readiness, rather than ranking candidates solely by cytotoxic activity. The synthesis also distinguishes promising combination strategies from single-agent activity and identifies potentially adverse biological responses that efficacy assessments based solely on primary tumor proliferation or apoptosis could overlook.

Nevertheless, interpretation is constrained by substantial heterogeneity among experimental models, treatment concentrations, exposure durations, outcome measures, and botanical preparations. The evidence base includes few primary experimental studies, often from individual laboratories, which limits assessment of independent reproducibility. Most mechanistic evidence remains *in vitro*, canine-specific PK information is limited, and spontaneous canine clinical evidence is sparse. Furthermore, the predominance of positive published findings raises the possibility of publication bias, but the small and heterogeneous evidence base precludes reliable quantitative assessment of its extent.

The findings for I3C warrant particular caution. In an orthotopic SCID mouse xenograft model of canine IMC, I3C reduced primary tumor proliferation but was associated with increased dermal and non-dermal tumor embolization and hepatic metastasis [52]. These adverse findings coincided with altered intratumoral steroid hormone concentrations, including increased estrone sulfate and estradiol. However, aromatase/*CYP19A1* upregulation was not directly demonstrated, and a causal endocrine mechanism for the increased metastatic dissemination has not been established.

Accordingly, the available evidence does not support the use of I3C as standalone therapy for advanced or inflammatory CMTs. Nevertheless, labeling I3C as definitively contraindicated in canine patients would overstate the evidence from a single immunodeficient murine xenograft model. Independent mechanistic investigation and validation in immunocompetent models are required before its safety and potential clinical relevance can be established [52].

Future research should prioritize candidates according to both biological activity and translational feasibility. Initial development should focus on standardized formulations, canine-specific pharmacokinetic and safety characterization, and confirmation that biologically active concentrations can be achieved in tumor tissue. Promising candidates should subsequently progress to biomarker-driven neoadjuvant window-of-opportunity studies in client-owned dogs with spontaneous CMTs, incorporating paired pretreatment and post-treatment specimens, pharmacokinetic measurements, tumor drug concentrations, pharmacodynamic biomarkers, and systematic safety assessment [17, 33, 110].

Combination strategies should be advanced only after their concentration-dependent interactions and pharmacokinetic compatibility are established *in vivo*. Botanical extracts require defined chemical fingerprints, quantitative marker specifications, batch-to-batch consistency, and appropriate manufacturing controls before clinical evaluation. Longer-term randomized studies should then determine whether pharmacodynamic activity translates into clinically meaningful improvements in recurrence, metastatic progression, quality-of-life, and survival.

Phytochemicals are promising experimental adjuncts, not established treatments, for CMTs. Their greatest potential may lie in rationally designed combination strategies and pharmacologically optimized formulations rather than empirical administration of poorly characterized botanical products. Translation will require progression from mechanistic *in vitro* evidence to standardized products, canine-specific pharmacokinetic and safety studies, demonstration of intratumoral target engagement, and appropriately designed clinical trials in dogs with spontaneous disease.

Spontaneous CMTs also provide a valuable comparative oncology platform because they arise naturally in immunocompetent animals and share selected histopathological, molecular, and metastatic characteristics with human breast cancer [14, 25, 33]. Rigorous evaluation of phytochemical-based interventions within a One Health framework could therefore improve evidence-based therapeutic options in veterinary oncology while generating complementary comparative data relevant to human breast cancer research.

DATA AVAILABILITY

All data analyzed during this study are included in this published article and its accompanying tables and figures. No primary raw datasets were generated during the current study.

GENERATIVE AI DECLARATION

During the preparation of this work, the authors used Google Gemini (Google LLC, USA) solely to enhance the resolution, font size, and visual clarity of Figure 2 based on the authors' original scientific diagram. After using this tool, the authors carefully reviewed and verified the visual and scientific accuracy of the figure and take full responsibility for the content of the publication. The author takes full responsibility for the scientific content and integrity of the figure and the entire manuscript. Generative AI technology was not used for study conception, literature screening, data analysis, or primary manuscript writing.

AUTHORS' CONTRIBUTIONS

KA is the sole author of this review article. KA: Conceptualized and designed the review, conducted the literature search, performed study screening and data extraction, analyzed and synthesized the findings, prepared the tables and figures, and drafted and critically revised the manuscript. The author has read and approved the final version of the manuscript.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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