RESEARCH PAPER

In-Vitro and In-Vivo Anticancer Activity of Hydroethanolic Root Extracts of Calotropis gigantea and Bauhinia variegata Against Ehrlich Ascites Carcinoma

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ABSTRACT

Background and Objective: Cancer continues to impose an enormous global burden, with inadequacies in conventional therapy necessitating the exploration of plant-derived alternatives. Calotropis gigantea and Bauhinia variegata are traditionally employed for tumor-related conditions in Ayurvedic medicine. This study investigated the in-vitro cytotoxic and in-vivo anticancer potential of their hydroethanolic root extracts against Ehrlich Ascites Carcinoma (EAC). **Materials and Methods:** In-vitro cytotoxicity was assessed using the MTT assay on EAC cells. In-vivo anticancer activity was evaluated in EAC-bearing Swiss albino mice (n = 6 per group) at doses of 100, 200, and 400 mg/kg (p.o., 14 days). Parameters included mean survival time (MST), percentage increase in life span (%ILS), packed cell volume (PCV), tumor cell count, hematological indices, serum biochemical markers, and in-vivo antioxidant status (SOD, CAT, GST, MDA). Cyclophosphamide (25 mg/kg, i.p.) served as the reference standard. Histopathological examination of liver and tumor tissues was performed. Acute oral toxicity was assessed per OECD Guideline 423. **Results:** CG showed greater in-vitro cytotoxicity (IC_{50} = 94.6 µg/mL) than BV (IC_{50} = 128.3 µg/mL). In-vivo, CG at 400 mg/kg increased MST to 32.1 ± 1.2 days (%ILS = 74.4%) and BV to 30.2 ± 1.2 days (%ILS = 64.1%), both significantly superior to EAC control (MST = 18.4 days; $p < 0.01$). Both extracts significantly reduced PCV, tumor cell count, and restored hematological and biochemical parameters ($p < 0.01$). BV demonstrated superior restoration of antioxidant enzymes (SOD, CAT, GST) and greater reduction of MDA. Histopathology confirmed near-normal liver architecture (CG) and moderate hepatoprotection (BV). No mortality or toxicity was observed up to 2000 mg/kg. **Conclusion:** C. gigantea exhibited stronger anticancer efficacy through cytotoxic and pro-apoptotic mechanisms, while B. variegata provided superior antioxidant enzyme restoration. Both extracts present complementary, multi-targeted therapeutic profiles warranting further molecular investigation.

Keywords: Calotropis gigantea, Bauhinia variegata, Ehrlich Ascites Carcinoma, MTT assay, anticancer, apoptosis, cardenolides, flavonoids, in-vivo antioxidant, mean survival time.

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1. INTRODUCTION

Cancer is a multifactorial malignancy characterized by uncontrolled cellular proliferation, evasion of apoptosis, and metastatic dissemination, representing the second leading cause of death globally. According to GLOBOCAN 2022, approximately 19.3 million new cancer cases were diagnosed worldwide, with projections estimating a rise to 28.4 million by 2040 (Bray et al., 2024). In India alone, the Indian Council of Medical Research (ICMR, 2023) reported over 1.4 million new cancer cases annually, with a disproportionately high mortality attributed to late-stage diagnosis, limited healthcare infrastructure, and unaffordability of advanced therapeutics.

Conventional cancer therapies, including cytotoxic chemotherapy, radiotherapy, and targeted agents, are limited by dose-limiting toxicities, multi-drug resistance (MDR), and prohibitive costs. The multi-drug resistance phenotype, mediated by over-expression of ABC efflux transporters (P-glycoprotein, MRP-1) and anti-apoptotic Bcl-2 family proteins, remains a principal cause of treatment failure (Gottesman, 2002). These limitations underscore the urgent need for novel, affordable, multi-

targeted therapeutic agents, particularly from natural product sources.

Plant-derived compounds contribute over 60% of currently approved anticancer drugs, including vincristine (Catharanthus roseus), paclitaxel (Taxus brevifolia), and camptothecin (Camptotheca acuminata), which modulate microtubule dynamics, topoisomerase activity, and apoptotic signaling respectively (Cragg & Newman, 2013). The multi-targeted nature of phytochemicals, enabling simultaneous modulation of NF-κB, PI3K/Akt, MAPK/ERK, and p53 pathways, confers advantages over single-target synthetic agents in the management of heterogeneous malignancies.

Calotropis gigantea (family Apocynaceae), revered as Arka in Ayurveda, is documented for use in tumor (Arbuda) management in classical texts. Its cardenolide constituents — particularly calotropin, calactin, and uscharin — inhibit Na^+/K^+-ATPase, triggering intracellular calcium accumulation, mitochondrial membrane depolarization, and caspase-mediated apoptosis. Modern preclinical studies confirm its cytotoxicity against multiple cancer cell lines (MCF-7, HeLa, HepG2, HT-29) with IC_{50} values in the 39–61 µg/mL range (Pathak et al., 2007; Wang et al., 2008).

Bauhinia variegata (family Fabaceae), known as Kanchanar, is the principal ingredient in Kanchanar Guggulu, prescribed for glandular swellings and lymphatic tumors. Rich in kaempferol, quercetin, rutin, and lupeol, its flavonoid and triterpenoid constituents modulate apoptotic pathways (Bax, caspase-3, Bcl-2), suppress NF-κB and HIF-1α signaling, and inhibit matrix metalloproteinases (MMP-2/9), collectively suppressing tumor invasion and angiogenesis (Mishra et al., 2013; Santos et al., 2018).

The present study is the first to provide a comprehensive comparative in-vivo evaluation of both plants against EAC-bearing mice, integrating survival analysis, tumor burden assessment, hematological and biochemical profiling, antioxidant enzyme quantification, and histopathological correlation, thereby establishing a mechanistic framework linking phytochemical composition with anticancer efficacy.

2. MATERIALS AND METHODS

2.1 Plant Authentication and Extract Preparation

Roots of C. gigantea and B. variegata were collected from Jaipur district, Rajasthan and authenticated by the Botanical Survey of India, Jodhpur (Certificate No. 1/2012/Tech./2024-25, dated 05/02/2025). Hydroethanolic extracts (70:30 v/v) were prepared by cold maceration (7 days), concentrated by rotary evaporation (40–45°C), and stored at 2–8°C. Extractive yields were 12.24% w/w (CG) and 14.92% w/w (BV).

2.2 Cell Line Procurement and Maintenance

Ehrlich Ascites Carcinoma (EAC) cells were procured from the National Centre for Cell Science (NCCS), Pune (Transaction No. 513415668121, dated 14/05/2025) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/mL), streptomycin (100 µg/mL), and amphotericin-B (2.5 µg/mL) at 37°C, 5% CO_2. Cell viability was confirmed ≥90% by trypan blue exclusion prior to each experiment.

2.3 In-Vitro Cytotoxicity: MTT Assay

EAC cells (1×10^4 cells/well) were seeded in 96-well plates and incubated for 24 hours. After treatment with extract concentrations (25–200 µg/mL), MTT reagent (5 mg/mL, 20 µL/well) was added and incubated for 4 hours. Formazan crystals were dissolved in DMSO (100 µL) and absorbance measured at 570 nm. Percentage cell viability and growth inhibition were calculated relative to vehicle-treated controls. IC_{50} values were determined by linear regression. DMSO concentration did not exceed 0.1%. Morphological changes were documented by inverted phase-contrast microscopy.

2.4 Experimental Animals and Ethical Compliance

Swiss albino mice (20–25 g, either sex) were procured from a CPCSEA-registered animal facility and maintained at 25 ± 2°C, 50–60% relative humidity, under a 12-hour light/dark cycle with ad libitum access to standard pellet diet and water. All experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC) and conducted in accordance with CPCSEA guidelines. Animals were acclimatized for 7 days prior to experimentation.

2.5 In-Vivo EAC Model and Experimental Design

EAC cells (1×10^6 viable cells in 0.2 mL) were inoculated intraperitoneally into mice on Day 0. Nine experimental groups (n = 6/group) were constituted: Group I (Normal control), Group II (EAC control), Group III (Cyclophosphamide 25 mg/kg i.p.), Groups IV–VI (CG at 100, 200, 400 mg/kg p.o.), and Groups VII–IX (BV at 100, 200, 400 mg/kg p.o.). Oral treatment commenced 24 hours post-inoculation and continued for 14 consecutive days. Doses were selected based on acute toxicity data (LD_{50} > 2000 mg/kg per OECD 423).

2.6 Assessment Parameters

Anticancer efficacy parameters included: (i) Mean Survival Time (MST = Σ survival days / n) and %ILS = $[(MST_{tp}{}^{eated} - MST^{dac\ coktrol}) / MST^{dac\ coktrol}] \times 100$; (ii) Body weight (Days 0, 5, 10, 15); (iii) Packed Cell Volume (PCV) by microhematocrit centrifugation; (iv) Viable tumor cell count by trypan blue exclusion. Hematological parameters (Hb, RBC, WBC) were estimated from retro-orbital blood samples. Serum biochemical markers (AST, ALT, ALP, urea, creatinine) were measured using commercial diagnostic kits. Liver and kidney tissues were homogenized for in-vivo antioxidant enzyme estimation: SOD (Kakkar et al., 1984), CAT (Aebi, 1984), GST, and

MDA (TBARS assay). Histopathological examination was performed on H&E-stained paraffin sections (5 µm).

2.7 Statistical Analysis

All results are expressed as Mean ± SEM. Statistical comparisons were performed using one-way ANOVA followed by Tukey's post-hoc multiple comparison test. $p < 0.05$ was considered statistically significant; $p < 0.01$ highly significant. Statistical analyses were performed using SPSS v20.0.

3. RESULTS

3.1 In-Vitro Cytotoxicity (MTT Assay)

CG demonstrated superior cytotoxic potency (IC_{50} = 94.6 µg/mL) vs. BV (IC_{50} = 128.3 µg/mL; cyclophosphamide = 18.7 µg/mL). At 200 µg/mL, CG reduced viability to 33.2 ± 1.4% (growth inhibition 66.8 ± 1.4%) and BV to 41.8 ± 1.5% (inhibition 58.2 ± 1.5%; both $p<0.01$ vs. control). Morphological examination showed dose-dependent cell shrinkage, cytoplasmic vacuolization, loss of membrane integrity, and reduced cellular density, consistent with apoptotic cell death, more pronounced in CG-treated cells.

Figure 1: In-Vitro Cytotoxic Activity of C. gigantea and B. variegata on EAC Cells (MTT Assay, Mean ± SEM, n=3)

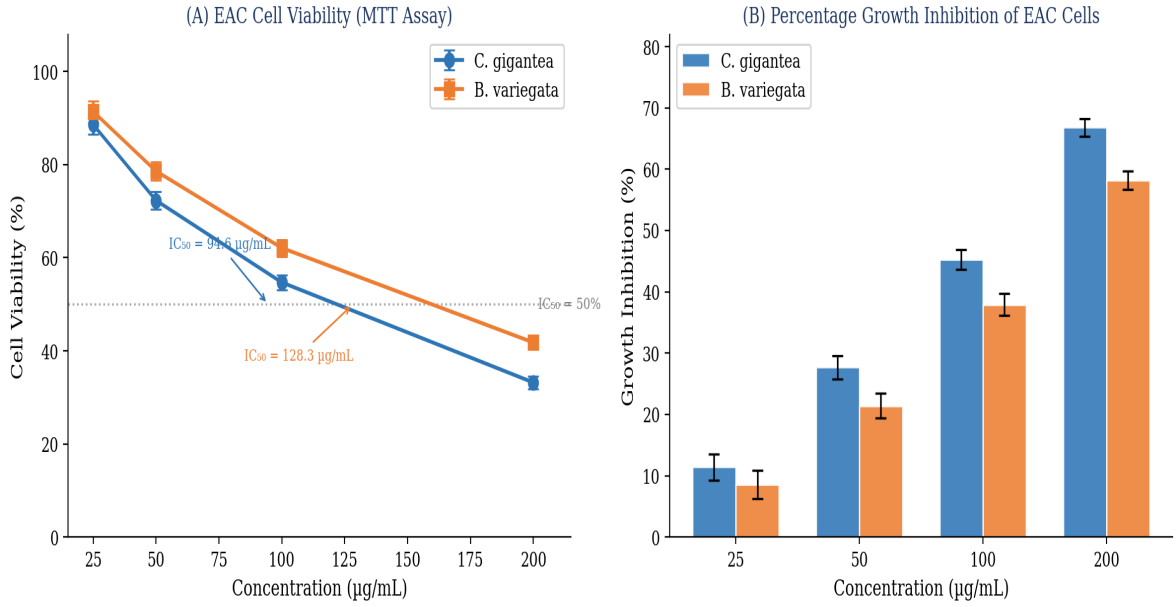

*Figure 1: In-vitro cytotoxic activity of C. gigantea and B. variegata on EAC cells. (A) Cell viability (MTT assay) and (B) percentage growth inhibition at concentrations of 25–200 µg/mL. Mean ± SEM (n=3). *p<0.05; **p<0.01 vs. control.*

3.2 In-Vivo Anticancer Activity

3.2.1 Mean Survival Time (MST) and %ILS

EAC control mice survived 18.4 ± 0.9 days. Treatment with CG at 400 mg/kg extended MST to 32.1 ± 1.2 days (%ILS = 74.4%), approaching the normal control (32.6 days) and the cyclophosphamide-treated group (MST = 34.1 ± 1.3 days; %ILS = 85.3%). BV at 400 mg/kg yielded MST = 30.2 ± 1.2 days (%ILS = 64.1%). All treated groups showed significant improvement over EAC control ($p < 0.05$ to $p < 0.01$, dose-dependent). These results are summarized in Table 1.

Table 1: Effect of Plant Extracts on MST, %ILS, PCV, and Tumor Cell Count in EAC-Bearing Mice

Group / Treatment	MST (days)	%ILS	PCV (mL/mouse)	Tumor Count (×10⁶/mL)
Normal Control	32.6 ± 1.2	—	—	—
EAC Control	18.4 ± 0.9	—	4.9 ± 0.18	92.6 ± 3.2
Cyclophosphamide (25 mg/kg)	34.1 ± 1.3**	85.3	1.8 ± 0.11**	30.5 ± 1.8**

CG 100 mg/kg	24.6 ± 1.0*	33.7	ND	ND
CG 200 mg/kg	28.9 ± 1.1**	57.1	ND	ND
CG 400 mg/kg	32.1 ± 1.2**	74.4	2.1 ± 0.12**	38.4 ± 2.1**
BV 100 mg/kg	23.1 ± 1.0*	25.5	ND	ND
BV 200 mg/kg	27.4 ± 1.1*	48.9	ND	ND
BV 400 mg/kg	30.2 ± 1.2**	64.1	2.6 ± 0.14*	46.7 ± 2.4*

*Values: Mean ± SEM (n = 6). *p < 0.05; **p < 0.01 vs. EAC control (one-way ANOVA, Tukey's test). ND: Not determined (only high-dose data shown for PCV and tumor count). CG: Calotropis gigantea; BV: Bauhinia variegata.*

3.2.2 Tumor Burden: Body Weight, PCV, and Tumor Cell Count

EAC control mice exhibited progressive body weight gain (18.4 to 33.4 g over 15 days) reflecting ascitic fluid accumulation. CG (400 mg/kg) and BV (400 mg/kg) significantly attenuated this gain (final weights: 26.1 ± 1.0 and 27.4 ± 1.1 g respectively; $p < 0.01$). PCV was reduced from 4.9 ± 0.18 mL (EAC control) to 2.1 ± 0.12 mL (CG; $p < 0.01$) and 2.6 ± 0.14 mL (BV; $p < 0.05$), approaching cyclophosphamide (1.8 ± 0.11 mL). Tumor cell count was reduced from 92.6 ± 3.2 × 10^6/mL (EAC control) to 38.4 ± 2.1 (CG; $p < 0.01$) and 46.7 ± 2.4 (BV; $p < 0.05$), indicating significant antiproliferative activity.

3.3 Hematological Parameters

EAC tumor progression induced anemia (Hb: 8.6 ± 0.3 g/dL), erythropenia (RBC: 3.4 ± 0.1 × 10^6/mm^3), and leukocytosis (WBC: 15.9 ± 0.5 × 10^3/mm^3). CG (400 mg/kg) significantly restored Hb (12.6 ± 0.4), RBC (5.8 ± 0.2), and WBC (8.6 ± 0.3; all $p<0.01$). BV (400 mg/kg) produced comparable restoration (Hb: 11.9 ± 0.3; RBC: 5.4 ± 0.2; WBC: 9.2 ± 0.4; all $p<0.05$), indicating myeloprotective activity.

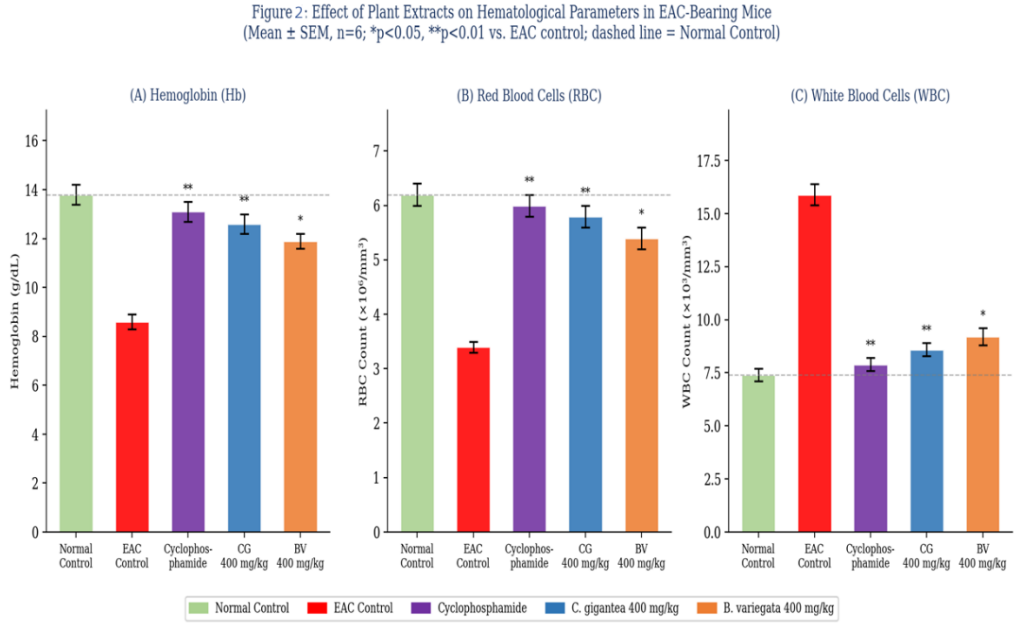

*Figure 2: Effect of C. gigantea and B. variegata (400 mg/kg) on hematological parameters — (A) Hemoglobin (g/dL), (B) Red Blood Cell count (×10^6/mm^3), and (C) White Blood Cell count (×10^3/mm^3) — in EAC-bearing mice. Dashed line = Normal control value. Mean ± SEM (n=6). *p<0.05; **p<0.01 vs. EAC control.*

3.4 Serum Biochemical Parameters

EAC tumor burden elevated hepatic enzymes (AST: 96.4 ± 2.9; ALT: 88.7 ± 2.6; ALP: 182.6 ± 5.1 U/L) and renal markers (urea: 68.4 ± 2.1 mg/dL; creatinine: 1.48 ± 0.05 mg/dL). CG (400 mg/kg) significantly normalized all markers ($p<0.01$); BV (400 mg/kg) produced comparable normalization ($p<0.05$), indicating hepatoprotective and nephroprotective effects.

Figure 3: Effect of Plant Extracts on Serum Biochemical Parameters in EAC-Bearing Mice
(Mean ± SEM, n=6; *p<0.05, **p<0.01 vs. EAC control)

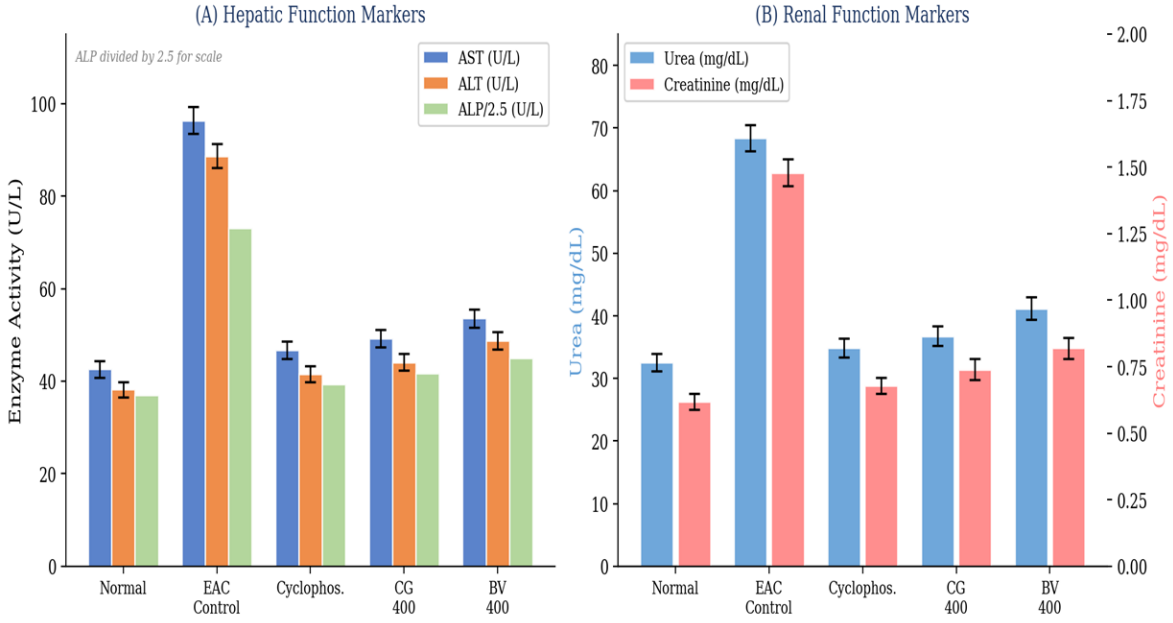

*Figure 3: Effect of plant extracts on serum biochemical parameters in EAC-bearing mice. (A) Hepatic function markers (AST, ALT, ALP) and (B) Renal function markers (urea and creatinine). Mean ± SEM (n=6). *p<0.05; **p<0.01 vs. EAC control.*

3.5 In-Vivo Antioxidant Status

EAC-induced oxidative stress significantly depleted SOD (4.12 ± 0.22 U/mg protein), CAT (31.8 ± 1.6 U/mg), and GST (3.24 ± 0.19 U/mg), while markedly elevating MDA (5.86 ± 0.18 nmol/mg). CG at 400 mg/kg restored SOD (7.84 ± 0.28), CAT (58.6 ± 2.0), GST (6.21 ± 0.26), and reduced MDA (2.62 ± 0.11) ($p < 0.01$). BV demonstrated comparable but slightly lower restoration of these parameters (SOD: 7.21 ± 0.26; CAT: 54.2 ± 1.9; GST: 5.78 ± 0.24; MDA: 2.94 ± 0.12; $p < 0.05$). Notably, BV exhibited superior antioxidant enzyme restoration relative to its cytotoxic potency, consistent with its polyphenol-rich composition. Table 2 summarizes these findings.

Table 2: In-Vivo Antioxidant Status in EAC-Bearing Mice Treated with Plant Extracts (400 mg/kg)

Group	MDA (nmol/mg)	SOD (U/mg)	CAT (U/mg)	GST (U/mg)
Normal Control	2.14 ± 0.09	8.92 ± 0.31	62.4 ± 2.1	6.84 ± 0.28
EAC Control	5.86 ± 0.18	4.12 ± 0.22	31.8 ± 1.6	3.24 ± 0.19
Cyclophosphamide	2.48 ± 0.10**	8.12 ± 0.29**	60.4 ± 2.1**	6.46 ± 0.27**
CG 400 mg/kg	2.62 ± 0.11**	7.84 ± 0.28**	58.6 ± 2.0**	6.21 ± 0.26**
BV 400 mg/kg	2.94 ± 0.12*	7.21 ± 0.26*	54.2 ± 1.9*	5.78 ± 0.24*

*Values: Mean ± SEM (n = 6). *p < 0.05; **p < 0.01 vs. EAC control. MDA: Malondialdehyde; SOD: Superoxide Dismutase; CAT: Catalase; GST: Glutathione S-Transferase.*

3.6 Histopathological Findings

Normal control liver sections exhibited regular hepatocellular cord architecture, well-delineated central veins, and normal sinusoidal spaces. EAC control

sections showed severe histopathological alterations including hepatocellular degeneration, sinusoidal dilatation, central vein congestion, inflammatory cell infiltration, cytoplasmic vacuolization, and focal necrosis, reflecting tumor-associated hepatotoxicity. CG (400 mg/kg) treatment resulted in near-normal hepatic architecture with significantly reduced inflammation and necrosis, indicating potent hepatoprotective activity. BV (400 mg/kg) demonstrated moderate restoration with mild residual degeneration. Tumor tissue from EAC control exhibited densely packed malignant cells with high nuclear-to-cytoplasmic ratio, pleomorphism, and hyperchromatic nuclei. CG-treated tumor sections showed marked reduction in cell density, increased cellular degeneration, and disrupted tumor architecture, whereas BV-treated sections revealed moderate reduction in tumor cell density, consistent with partial growth suppression.

4. DISCUSSION

The present study provides the first comprehensive comparative evaluation of the in-vitro cytotoxic and in-vivo anticancer activities of C. gigantea and B. variegata hydroethanolic root extracts against EAC, integrating pharmacological, biochemical, antioxidant, and histopathological end-points.

The superior in-vitro cytotoxicity of CG (IC_{50} = 94.6 µg/mL) over BV (IC_{50} = 128.3 µg/mL) is mechanistically attributed to its cardenolide glycoside content. Calotropin and calactin inhibit the ubiquitous cellular Na^+/K^+-ATPase pump, leading to intracellular calcium overload, mitochondrial membrane potential ($\Delta\Psi m$) collapse, cytochrome c release, and sequential activation of the caspase cascade (caspase-9 → caspase-3), culminating in apoptotic cell death. This mechanism is analogous to cardiac glycosides (digitoxin, ouabain) but with selective cytotoxicity toward cancer cells, possibly due to altered Na^+/K^+-ATPase isoform expression in transformed cells (Hoopes et al., 2018). The terpenoid constituents (lupeol, β-amyrin) further contribute through mitochondrial permeabilization and modulation of the Bcl-2/Bax ratio, reinforcing intrinsic apoptotic pathway activation.

The in-vivo efficacy of CG (400 mg/kg; %ILS = 74.4%) closely approached that of cyclophosphamide (%ILS = 85.3%), demonstrating translational relevance of the in-vitro findings. The significant reduction in PCV (57.1% decrease vs. EAC control) and tumor cell count (58.5% decrease) confirms antiproliferative activity in the ascitic milieu. BV's lower but significant in-vivo efficacy (%ILS = 64.1%) likely reflects its flavonoid-dominant mechanism, wherein kaempferol and quercetin modulate pro-apoptotic PI3K/Akt and p53 pathways rather than direct membrane-disrupting cytotoxicity. The quercetin-mediated inhibition of NF-κB translocation further reduces expression of anti-apoptotic genes (Bcl-2, XIAP) and angiogenic factors (VEGF, MMP-9), contributing to tumor suppression.

The normalization of hematological parameters by both extracts — restoration of Hb, RBC, and WBC toward normal values — indicates myeloprotective activity potentially mediated by reduction of tumor-associated cytokine-driven myelosuppression. The significant restoration of serum AST, ALT, ALP, urea, and creatinine suggests both hepatoprotective and nephroprotective activities, likely through antioxidant-mediated membrane stabilization and reduction of lipid peroxidation in organ tissues.

The in-vivo antioxidant analysis reveals a mechanistically important dissociation: CG demonstrated superior tumor suppression while BV showed superior antioxidant enzyme restoration. This suggests that CG's anticancer mechanism is primarily driven by pro-apoptotic cytotoxic phytoconstituents (cardenolides, terpenoids) rather than antioxidant-mediated pathways, whereas BV's therapeutic effect operates through polyphenol-driven redox modulation, Nrf2 pathway activation, and secondary suppression of oxidative stress-driven tumor promotion. These complementary profiles are consistent with the "redox paradox" concept, wherein cancer cells maintaining elevated ROS for proliferative signaling can be targeted either by ROS-generating cytotoxic agents (CG) or by antioxidant restoration strategies that reverse ROS-mediated immunosuppression and DNA damage (BV).

The histopathological findings corroborate these mechanistic interpretations: near-normal liver architecture in CG-treated mice reflects potent anti-inflammatory activity (COX-2, NF-κB inhibition) and rapid tumor regression reducing systemic toxin release, while moderate hepatoprotection in BV-treated mice aligns with its antioxidant membrane-stabilizing triterpenoid (lupeol, oleanolic acid) content. The absence of toxicity at doses up to 2000 mg/kg supports the safety of both extracts, with a favorable therapeutic window for pharmacological development.

5. CONCLUSION

This study demonstrates that C. gigantea and B. variegata hydroethanolic root extracts possess significant anticancer activity against EAC through distinct but complementary mechanisms. C. gigantea exhibited superior cytotoxic and in-vivo tumor suppressive efficacy driven by cardenolide- and terpenoid-mediated pro-apoptotic signaling, including Na^+/K^+-ATPase inhibition and mitochondrial dysfunction. B. variegata provided potent antioxidant enzyme restoration and moderate tumor suppression through flavonoid-mediated redox modulation and NF-κB/PI3K pathway inhibition. Both extracts restored hematological indices and organ function markers, affirming systemic myeloprotective and organoprotective activity. The

complementary pharmacodynamic profiles of CG and BV suggest potential synergism in polyherbal anticancer formulations. Future investigations should focus on bioactivity-guided isolation of active cardenolides and flavonoids, molecular docking, and evaluation in genetically defined tumor models to facilitate clinical translation.

Declarations

Ethics Approval: All animal experiments were conducted in accordance with CPCSEA guidelines and approved by the Institutional Animal Ethics Committee (IAEC), Bhupal Nobles' University, Udaipur.

Cell Line Acknowledgement: EAC cell line was procured from the National Centre for Cell Science (NCCS), Pune, India (Transaction No. 513415668121). NCCS is duly acknowledged.

Conflict of Interest: The authors declare no conflict of interest.

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