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In vitro cytotoxic evaluation of *Blaptica dubia* (Dubia roach) hemolymph against MCF-7 human breast adenocarcinoma cells: Elucidating the pro-apoptotic and anti-proliferative mechanisms

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Abstract

Background: Breast cancer remains the leading cause of cancer-related deaths among women globally. Despite therapeutic advances, drug resistance and toxicity necessitate discovery of novel bioactive compounds from underexplored natural sources. Insects, particularly cockroaches, produce hemolymph rich in antimicrobial peptides with potential anticancer properties. *Blaptica dubia* (Dubia roach) is widely cultured as feeder insect but its hemolymph has never been evaluated for anticancer activity.

Objective: To investigate the *in vitro* anticancer activity of *Blaptica dubia* hemolymph against MCF-7 human breast adenocarcinoma cells and assess its selectivity toward normal cells.

Methods: Hemolymph was collected from adult *B. dubia* (n=50) by cold immobilization and leg amputation. Cell-free hemolymph was obtained by centrifugation (4,000×g, 15 min, 4°C), filter-sterilized, and protein concentration quantified (Bradford assay). MCF-7 cells and normal human dermal fibroblasts (NHDF) were treated with hemolymph at 25–400 µg/mL for 24, 48, and 72 hours. Cytotoxicity was assessed by MTT assay. Apoptosis was evaluated using acridine orange/ethidium bromide (AO/EB) dual staining and caspase-3 colorimetric assay. Cell cycle distribution was analyzed by propidium iodide flow cytometry.

Results: *B. dubia* hemolymph exhibited significant dose- and time-dependent cytotoxicity against MCF-7 cells. The 48-hour IC50 was 112.4 µg/mL. At 400 µg/mL, viability decreased to 21.4% (MCF-7) versus 72.3% (NHDF), yielding a selectivity index of 4.25. AO/EB staining revealed typical apoptotic morphology: chromatin condensation, nuclear fragmentation, and apoptotic bodies (apoptotic index 54.7% vs. 4.2% control). Caspase-3 activity increased 4.2-fold at 200 µg/mL ($p<0.001$). Flow cytometry showed G0/G1 arrest (68.2% vs. 48.5% control) and increased sub-G1 fraction (22.4% vs. 2.3%).

Conclusion: *Blaptica dubia* hemolymph selectively induces caspase-dependent apoptosis and G0/G1 cell cycle arrest in MCF-7 cells. This is the first report of anticancer activity from *B. dubia*, establishing it as a promising source for bioprospecting novel anticancer lead compounds. Further fractionation and in *vivo* studies are warranted.

Keywords: *Blaptica dubia*, Dubia roach, hemolymph, MCF-7, breast cancer, apoptosis, cell cycle arrest, natural product

Introduction

1. Global Burden of Breast Cancer

Breast cancer is the most frequently diagnosed malignancy and the leading cause of cancer mortality among women worldwide. According to the Global Cancer Observatory (GLOBOCAN 2022), approximately 2.3 million new cases and 685,000 deaths were attributed to breast cancer in 2020 alone. These numbers are projected to rise to over 3 million new cases annually by 2040 due to aging populations, lifestyle changes, and improved diagnostic capabilities in low- and middle-income countries. The age-standardized incidence rate varies from 27 per 100,000 in South-Central Asia to 92 per 100,000 in North America, reflecting differences in screening, genetic predisposition, reproductive factors, and environmental exposures.

Among breast cancer subtypes, hormone receptor-positive (HR+)/human epidermal growth factor receptor 2-negative (HER2–) tumours account for approximately 70% of all cases. The MCF-7 cell line, derived from a pleural effusion of a patient with metastatic breast cancer in 1970, remains the most extensively studied model for HR+ breast cancer. MCF-7 cells express estrogen receptor alpha (ERα), progesterone receptor (PR), and wild-type p53, making them responsive to endocrine therapies such as tamoxifen and aromatase inhibitors. However, acquired resistance to these agents develops in up to 40% of patients within 5–10 years of treatment, leading to disease progression and mortality.

Fig 1: *Blaptica dubia* (Dubia Roach)

2. Limitations of Current Therapies

Conventional breast cancer treatments include surgery, radiation, chemotherapy (e.g., doxorubicin, cyclophosphamide, paclitaxel), endocrine therapy (tamoxifen, fulvestrant, letrozole), and targeted therapy (trastuzumab, pertuzumab, everolimus, palbociclib). Despite significant improvements in patient outcomes, several limitations persist: (i) Severe adverse effects including cardiotoxicity (doxorubicin), neuropathy (paclitaxel), thromboembolism (tamoxifen), and interstitial lung disease (everolimus); (ii) Primary and acquired resistance mediated by mutations in ESR1, activation of alternative growth pathways (PI3K/AKT/mTOR), and upregulation of drug efflux pumps (ABCB1/P-glycoprotein); (iii) High financial toxicity, with novel targeted therapies costing over $10,000 per month, placing them beyond reach for patients in low-resource settings; (iv) Limited efficacy against triple-negative breast cancer (TNBC). These challenges underscore the urgent need for novel, affordable, and less toxic anticancer agents [1].

3. Natural Products as Sources of Anticancer Agents

Natural products have historically been the most productive source of anticancer drugs. According to Newman and Cragg (2020), over 60% of approved anticancer agents between 1981 and 2019 were either natural products or directly derived from them. Notable examples include paclitaxel (from the Pacific yew tree *Taxus brevifolia*), vincristine and vinblastine (from the Madagascar periwinkle *Catharanthus roseus*), camptothecin (from the Chinese tree *Camptotheca acuminata*), and doxorubicin (from *Streptomyces peucetius*). These compounds continue to inspire synthetic and semi-synthetic analogs with improved pharmacological profiles.

However, plant-based drug discovery faces challenges: slow growth rates, endangered species status, low yields of active compounds, and dependence on agricultural land and climate. Marine organisms, another rich source, require specialized collection and culturing infrastructure. Insects, despite comprising over 80% of described animal species, remain vastly underexplored in pharmaceutical discovery. This represents a significant missed opportunity, as insects have evolved sophisticated innate immune systems over 400 million years, producing a diverse arsenal of antimicrobial and cytotoxic molecules to defend against pathogens and parasites [2].

4. Insect Hemolymph: Composition and Bioactivity

Insect hemolymph is the circulatory fluid equivalent to blood and lymph in vertebrates. It consists of a liquid plasma containing water, proteins (enzymes, storage proteins, lipophorins, transferrins), carbohydrates (trehalose), lipids, ions, and cellular components (hemocytes). Unlike vertebrate blood, insect hemolymph lacks erythrocytes and hemoglobin, relying on tracheal systems for oxygen transport. The immune function of hemolymph is mediated by: (i) Cellular responses – phagocytosis, nodulation, encapsulation by hemocytes; (ii) Humoral responses – production of antimicrobial peptides (AMPs), lysozymes, lectins, prophenoloxidase cascade, and reactive oxygen/nitrogen species.

Over 300 insect-derived AMPs have been characterized, belonging to families such as cecropins (α-helical, from Lepidoptera and Diptera), defensins (cysteine-rich, from many orders), attacins (glycine-rich, from Lepidoptera), diptericins (proline-rich, from Diptera), and thanatins (from *Galleria mellonella*). Several insect AMPs exhibit anticancer activity by selectively disrupting cancer cell membranes due to their higher negative surface charge and increased membrane fluidity compared to normal cells [3].

5. Previous Studies on Cockroach Hemolymph

Cockroaches (order Blattodea) have attracted research interest due to their remarkable resilience, longevity, and resistance to pathogens. Hemolymph from several cockroach species has been shown to possess bioactivities:

- ***Periplaneta americana* (American cockroach):** Hemolymph and its fractions exhibited cytotoxicity against HepG2 (hepatocellular carcinoma), A549 (lung adenocarcinoma), and MCF-7 cells. The active component was identified as a 12-kDa peptide with sequence similarity to cecropin. Additionally, *P. americana* hemolymph induced apoptosis in gastric cancer cells via mitochondrial pathway (Bax/Bcl-2 dysregulation, cytochrome c release, caspase-9 and -3 activation).

- ***Blaberus craniifer* (Death's head cockroach):** Hemolymph showed antimicrobial and anticancer activity against HeLa (cervical) and Jurkat (T-cell leukemia) cells, with IC50 values around 130–150 µg/mL. Activity was heat-stable and protease-sensitive, suggesting peptidic nature.

- ***Gromphadorhina portentosa* (Madagascar hissing cockroach):** Hemolymph induced necrosis in PC-3 prostate cancer cells at high concentrations (IC50 300 µg/mL) but showed less selectivity.

- ***Blattella germanica* (German cockroach):** Hemolymph-derived peptides demonstrated antifungal and moderate anticancer activity.

Despite these promising reports, no study to date has investigated *Blaptica dubia* (Dubia roach), a species extensively farmed as reptile feed. *B. dubia* belongs to the family Blaberidae, same as *B. craniifer*, but differs in ecology (tropical Central/South America vs. Central America/Caribbean), reproduction (ovoviviparous with extended brood care), and diet (omnivorous but prefers fruits and vegetable matter). These differences may result in distinct hemolymph composition and bioactivity [4].

Materials and Methods

1. Collection and Maintenance of *Blaptica dubia*

Adult *Blaptica dubia* roaches (4–6 months old, both sexes, body weight 2.5–3.5 g) were obtained from a certified Babu Mahipati Singh Mahavidyalaya Tiloi, Amethi UP, India. Upon arrival, roaches were transferred to ventilated plastic storage containers (60 L × 40 W × 40 H cm) in Chemistry Lab BMS College f Pharmacy Amethi, UP lined with egg

crates as harborage. The containers were maintained in a climate-controlled room at 28 ± 2°C, 60 ± 5% relative humidity, with a 12:12 hour light: dark cycle. Roaches were fed ad libitum with a diet consisting of 70% chicken mash (Purina Mills, 22% protein), 20% rolled oats, and 10% dried milk powder. Fresh oranges (cut into wedges) were provided three times per week as a water source and vitamin supplement. Water gel crystals (Zilla, Inc.) were provided continuously to prevent dehydration. Containers were cleaned weekly to remove frass and exuviae (molted skins). Health status was monitored daily; any dead or moribund roaches were removed immediately. Roaches were acclimated for two weeks prior to hemolymph collection. For 24 hours before hemolymph collection, food was withheld to reduce gut contamination of hemolymph, but water gel was provided.

2. Hemolymph Collection

Hemolymph collection was performed using a cold immobilization method approved for invertebrate use (no vertebrate animal ethics required). Roaches (n=50) were individually transferred to a clean plastic container and placed on crushed ice for 15 minutes until complete immobility was achieved. Immobilization was confirmed by absence of antennal movement and leg withdrawal upon gentle prodding. Using sterile micro-scissors (Fine Science Tools, 15000-08), one midleg (third pair of legs) was amputated at the coxa-trochanter joint. A droplet of hemolymph (typically 50–100 µL per roach) appeared at the amputation site within 5–10 seconds.

Hemolymph was collected into ice-cold sterile 1.5 mL microcentrifuge tubes (Eppendorf) containing 10 µL of 1 mM phenylthiourea (PTU, Sigma-Aldrich, P7629) dissolved in phosphate-buffered saline (PBS, pH 7.4). PTU acts as a tyrosinase inhibitor, preventing melanization (phenoloxidase-mediated conversion of tyrosine to melanin) that would otherwise coagulate the hemolymph and inactivate bioactive proteins. Hemolymph was collected from each roach for a maximum of 2 minutes; after collection, the roach was transferred to a recovery container with food and water gel at 25°C. Leg amputation is non-lethal, and the leg regenerates over subsequent molts (roaches undergo 7–9 instars before adulthood; adults do not molt). No roaches died during or after collection. All procedures were performed under sterile laminar flow hood (Class II, Thermo Scientific) to prevent microbial contamination [5].

3. Hemolymph Processing and Sterilization

Pooled hemolymph from 50 roaches (total volume approximately 3.2 mL) was immediately centrifuged at 4,000 × g for 15 minutes at 4°C using a refrigerated centrifuge (Eppendorf 5810R). This centrifugation step removes haemocytes (insect blood cells) and cellular debris, yielding cell-free hemolymph (CFH). The supernatant was carefully aspirated without disturbing the pellet. To remove any remaining particulates and ensure sterility, the CFH was passed through a 0.22 µm syringe filter (Millex-GV, Millipore Sigma, SLGV033RS). Filter-sterilized CFH was aliquoted into 100 µL portions in sterile cryovials (Corning). Aliquots were flash-frozen in liquid nitrogen and stored at −80°C until use. A pilot study confirmed that frozen storage for up to 3 months did not reduce bioactivity (data not shown). For each experiment, a fresh aliquot was thawed on ice, used immediately, and any leftover was discarded (no freeze-thaw cycles).

4. Protein Quantification by Bradford Assay

The total protein concentration of *B. dubia* CFH was determined using the Bradford method (Bradford, 1976) with Bio-Rad Protein Assay Dye Reagent (Bio-Rad, 5000006). Bovine serum albumin (BSA, Sigma, A9418) was used as a standard. Standard curves were prepared by serial dilution of BSA (2 mg/mL stock) in PBS to obtain final concentrations of 0, 25, 50, 100, 200, 400, 800, and 1,000 µg/mL. CFH samples were diluted 1:10 and 1:20 in PBS. In triplicate wells of a 96-well plate, 10 µL of each standard or diluted sample was mixed with 200 µL of Bradford reagent (diluted 1:5 with distilled water). After 5 minutes of incubation at room temperature in the dark, absorbance was measured at 595 nm using a microplate reader (Bio Tek ELx800, Agilent). Protein concentration was calculated from the linear portion of the BSA standard curve ($R^2 > 0.995$). The assay was repeated three times independently. The mean protein concentration was used to standardize hemolymph concentrations for all subsequent cell-based assays: 25, 50, 100, 200, 300, and 400 µg protein/mL. For simplicity, these are referred to as "hemolymph concentrations" throughout [6].

5. Cell Lines and Culture Conditions

Two human cell lines were used: MCF-7 human breast adenocarcinoma (ATCC HTB-22™) and normal human dermal fibroblasts (NHDF, ATCC PCS-201-012). Both were obtained from 200 Bed Hospital Raebareli UP. MCF-7 cells were cultured in BMSCOP Amethi UP 1640 medium (Gibco, 11875093) supplemented with 10% fetal bovine serum (FBS, HyClone, SH30070.03), 1% penicillin-streptomycin solution (100 U/mL penicillin and 100 µg/mL streptomycin, Gibco, 15140122), and 0.01 mg/mL human recombinant insulin (Sigma, I9278). NHDF cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, 11965092) supplemented with 10% FBS and 1% antibiotic-antimycotic (Gibco, 15240062).

Both cell lines were maintained in 75 cm² cell culture flasks (Corning, #430641) at 37°C in a humidified atmosphere of 5% CO_2 (Thermo Scientific Hera cell 150i). Medium was changed every 48–72 hours. Cells were subculture at 80–90% confluence using 0.25% trypsin-EDTA solution (Gibco, #25200056). Briefly, cells were washed twice with PBS, incubated with 2 mL trypsin-EDTA for 3–5 minutes at 37°C until cells detached, neutralized with 8 mL complete medium, centrifuged at 200 × g for 5 minutes, resuspended in fresh medium, and seeded at split ratios of 1:3 to 1:6. Cells were regularly tested for mycoplasma contamination using PCR-based detection (Look Out Mycoplasma PCR Detection Kit, Sigma, MP0035). Only mycoplasma-negative cultures between passages 5 and 12 were used for experiments.

6. MTT Cytotoxicity Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a colorimetric method that measures the reduction of yellow tetrazolium

salt to purple formazan crystals by mitochondrial succinate dehydrogenase in metabolically active cells. Thus, absorbance is directly proportional to the number of viable cells.

Cell seeding: MCF-7 and NHDF cells were harvested during exponential growth phase and counted using a hemacytometer (Hausser Scientific) with trypan blue exclusion (viability >95% required). Cells were seeded at a density of 1×10^4 cells per well in 96-well flat-bottom plates (Corning, #3599) containing 100 µL of respective complete medium. Plates were incubated at 37°C, 5% CO_2 overnight (16–18 hours) to allow cell adhesion in Microbiology Lab in BMS College of Pharmacy Tiloi, Amethi UP India.

Treatment: After adhesion, the medium was aspirated and replaced with 100 µL of fresh medium containing *B. dubia* hemolymph at final protein concentrations of 0 (vehicle control: PBS only), 25, 50, 100, 200, 300, and 400 µg/mL. Each concentration was tested in six replicate wells. Positive control wells received 1 µM doxorubicin hydrochloride (Sigma, D1515, dissolved in DMSO, final DMSO concentration ≤0.01%). Negative (vehicle) control wells received PBS equal to the volume used for hemolymph (no hemolymph). Blank wells contained medium only (no cells) to correct for background absorbance. Plates were incubated for 24, 48, or 72 hours.

MTT addition and formazan solubilization: At each endpoint, 10 µL of MTT solution (5 mg/mL in sterile PBS, Sigma, M5655) was added to each well (final concentration 0.5 mg/mL). Plates were returned to the incubator for 4 hours. During this period, viable cells converted MTT to insoluble purple formazan. After 4 hours, the medium was carefully aspirated, and 100 µL of DMSO (Sigma, D8418) was added to each well to dissolve the formazan crystals. Plates were gently shaken on an orbital shaker (100 rpm, 10 minutes, room temperature) to ensure complete dissolution.

Absorbance measurement: Absorbance was read at 570 nm with a reference wavelength of 630 nm using a microplate reader (Bio Tek ELx800). Percent cell viability was calculated as:

$$\% \text{ Viability} = [(OD_{570} - OD_{630})\text{ treated} - (OD_{570} - OD_{630})\text{ blank}] / [(OD_{570} - OD_{630})\text{ control} - (OD_{570} - OD_{630})\text{ blank}] \times 100$$

The half-maximal inhibitory concentration (IC50) was determined by non-linear regression analysis (log(inhibitor) vs. normalized response, variable slope) using GraphPad Prism version 9.5.0 (GraphPad Software, San Diego, CA, USA). All experiments were performed in three independent biological replicates (each with six technical replicates). Results were expressed as mean ± standard deviation (SD).

7. Selectivity Index Calculation

The selectivity index (SI) quantifies the relative toxicity of a compound toward cancer cells versus normal cells. An SI > 3 is generally considered indicative of selective anticancer activity, while SI > 10 suggests high selectivity. SI was calculated using the formula:

SI = IC50 (normal cells) / IC50 (cancer cells)

Where IC50 (normal cells) was determined from the MTT assay on NHDF fibroblasts at 48 hours, and IC50 (cancer cells) from MCF-7 cells at the same time point [7].

8. Morphological Assessment of Apoptosis Using Acridine Orange/Ethidium Bromide (AO/EB) Dual Staining

AO/EB staining distinguishes viable, early apoptotic, late apoptotic, and necrotic cells based on membrane integrity and nuclear morphology. Acridine orange (AO) permeates both viable and non-viable cells, intercalating into DNA (green fluorescence) and RNA (red fluorescence). Ethidium bromide (EB) is excluded by viable cells but enters cells with compromised membranes, intercalating into DNA (orange-red fluorescence). EB also quenches AO fluorescence via fluorescence resonance energy transfer (FRET) when both are present.

Procedure: MCF-7 cells (5×10^4 cells/well) were seeded on sterile 12 mm circular coverslips (Fisher Scientific, #12-545-80) placed in 6-well plates (Corning, #3516). After overnight adhesion, cells were treated with fresh medium containing either vehicle control (PBS) or *B. dubia* hemolymph at the previously determined 48-hour IC50 concentration (112.4 µg/mL). After 48 hours of incubation, the medium was removed, and cells were washed twice with ice-cold PBS. A staining solution containing 100 µg/mL AO and 100 µg/mL EB (both Sigma-Aldrich, A6014 and E8751) in PBS was freshly prepared. One hundred microliters of staining solution was added to each coverslip, and plates were incubated at room temperature in the dark for 5 minutes. Coverslips were then inverted onto glass slides (Fisher Scientific) and immediately examined under a fluorescence microscope (Olympus BX53, equipped with FITC filter: excitation 488 nm, emission 520 nm; and TRITC filter: excitation 540 nm, emission 590 nm). For each coverslip, five randomly selected fields (minimum 200 cells total) were photographed. Cell classification:

- **Viable:** Uniform green nucleus, intact nuclear structure.
- **Early apoptotic:** Bright green nucleus with condensed or fragmented chromatin (margination), often "beaded" appearance.
- **Late apoptotic:** Orange-red nucleus with condensed/fragmented chromatin; membrane blebbing may be present.
- **Necrotic:** Uniform orange-red nucleus without chromatin fragmentation; swollen cell morphology.

The apoptotic index was calculated as: (number of early apoptotic + late apoptotic cells) / total cells counted × 100. The experiment was performed in triplicate.

9. Caspase-3 Activity Colorimetric Assay

Caspase-3 is a key executioner caspase in both intrinsic (mitochondrial) and extrinsic (death receptor) apoptotic pathways. Its activation is a hallmark of irreversible commitment to apoptosis. Caspase-3 activity was measured using a colorimetric assay kit (Abcam, ab39401) based on cleavage of the synthetic substrate DEVD-p-nitroaniline

(DEVD-pNA). Upon cleavage, free pNA releases a yellow colour measurable at 405 nm.

Cell treatment and lysis: MCF-7 cells (1×10^6 cells per condition) were seeded in 60 mm dishes (Corning, #430166) and treated with hemolymph at concentrations of 0 (control), 50, 112.4 (IC50), and 200 µg/mL for 48 hours. After treatment, cells were harvested by trypsinization, washed once with ice-cold PBS, and centrifuged at 800 × g for 5 minutes at 4°C. The cell pellet was resuspended in 50 µL of ice-cold cell lysis buffer (provided with kit) supplemented with 1 mM dithiothreitol (DTT, added fresh) to preserve caspase activity. Cells were incubated on ice for 10 minutes with gentle vortexing every 2 minutes. Lysates were centrifuged at 10,000 × g for 5 minutes at 4°C to remove insoluble debris. The supernatant (protein lysate) was transferred to fresh tubes.

Protein quantification of lysates: Protein concentration of each lysate was determined using the Bradford assay as described in section 2.4, to ensure equal protein loading in the caspase assay.

Caspase-3 assay: For each sample, 50 µg of total protein was diluted to 50 µL with lysis buffer in a 96-well plate well. Fifty microliters of 2× reaction buffer (containing 10 mM DTT) was added, followed by 5 µL of 4 mM DEVD-pNA substrate (final concentration 200 µM). A blank well (no lysate, only buffer and substrate) and a positive control (recombinant human caspase-3, provided with kit) were included. Plates were incubated at 37°C for 2 hours in the dark. Absorbance was read at 405 nm using a microplate reader. Fold increase in caspase-3 activity was calculated as: (OD_{405} treated – OD_{405} blank) / (OD_{405} control – OD_{405} blank). The assay was performed in triplicate independent experiments, each with duplicate technical replicates.

10. Cell Cycle Analysis by Flow Cytometry

Propidium iodide (PI) staining of DNA allows quantification of cellular DNA content by flow cytometry, distinguishing cells in G0/G1 (2N DNA), S (intermediate DNA synthesis), and G2/M (4N DNA) phases. Cells with sub-G1 DNA content (hypodiploid) are characteristic of apoptotic cells with DNA fragmentation.

Cell treatment and fixation: MCF-7 cells (5×10^5 per well in 6-well plates) were treated with either vehicle control (PBS) or *B. dubia* hemolymph at 112.4 µg/mL (48-hour IC50) for 48 hours. After treatment, adherent cells were trypsinized, combined with floating cells from the supernatant (to include apoptotic cells that have detached), and pooled. Cells were centrifuged at 800 × g for 5 minutes, washed twice with ice-cold PBS, and resuspended in 300 µL PBS. While vortexing gently, 700 µL of ice-cold 100% ethanol was added dropwise to achieve a final concentration of 70% ethanol. Cells were fixed at –20°C overnight (minimum 12 hours). Fixed cells can be stored at –20°C for up to 1 month [8].

PI staining: Fixed cells were centrifuged at 800 × g for 5 minutes, washed twice with PBS to remove ethanol, and resuspended in 500 µL of PI staining solution containing: 50 µg/mL propidium iodide (Sigma, P4170), 100 µg/mL RNase A (Thermo Scientific, EN0531), and 0.1% Triton X-100 (Sigma, T8787) in PBS. RNase A is essential to degrade RNA, which otherwise binds PI and causes overestimation of DNA content. Triton X-100 permeabilizes cell membranes. Cells were incubated at 37°C for 30 minutes in the dark.

Flow cytometry acquisition and analysis: Stained cells were analyzed using a BD FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 488 nm argon laser and a 585/42 nm bandpass filter (PE channel) for PI detection. At least 20,000 events were acquired per sample at a low flow rate (≤200 events/sec) to ensure high-resolution DNA histograms. Doublets and aggregates were excluded by gating on FSC-A vs. FSC-H (area/height). The cell cycle distribution (percentage of cells in sub-G1, G0/G1, S, and G2/M phases) was determined using ModFit LT software version 5.0 (Verity Software House, Topsham, ME, USA) with the Dean-Jett-Fox model for cell cycle fitting. Three independent biological replicates were performed [9].

11. Statistical Analysis

All quantitative data are presented as mean ± standard deviation (SD) of at least three independent biological replicates (n=3, unless otherwise specified). Normality of data distribution was assessed using the Shapiro-Wilk test. Homogeneity of variances was tested using Levene's test. For comparisons between two groups (treated vs. control), the unpaired two-tailed Student's t-test was used when data were normally distributed; otherwise, the Mann-Whitney U test was applied. For comparisons among multiple groups (different hemolymph concentrations or time points), one-way analysis of variance (ANOVA) was performed, followed by Tukey's honestly significant difference (HSD) post-hoc test for multiple comparisons when ANOVA indicated significant differences ($p < 0.05$). For two-way factorial designs (concentration × time), two-way ANOVA was used. All statistical analyses were performed using GraphPad Prism 9.5.0 (GraphPad Software, San Diego, CA) and IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY). A p-value less than 0.05 was considered statistically significant. Significance levels are indicated as: ns (not significant, $p \geq 0.05$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

12. Quality Control and Reproducibility Measures

To ensure reproducibility and minimize experimental bias, the following measures were implemented: (i) All experiments were blinded by coding hemolymph and control samples using a randomization list generated by a researcher not involved in data acquisition; (ii) Hemolymph batches were characterized for protein concentration and baseline activity against MCF-7 cells before use; only batches with IC50 within 10% of the mean were used; (iii) Cell culture passages were limited to 5–12 to avoid phenotypic drift; (iv) MTT assays included vehicle controls, positive controls, and blank wells; (v) All flow cytometry and fluorescence microscopy analyses were performed by operators blinded to treatment groups; (vi) Raw data were archived and re-analyzed by a second independent researcher for verification.

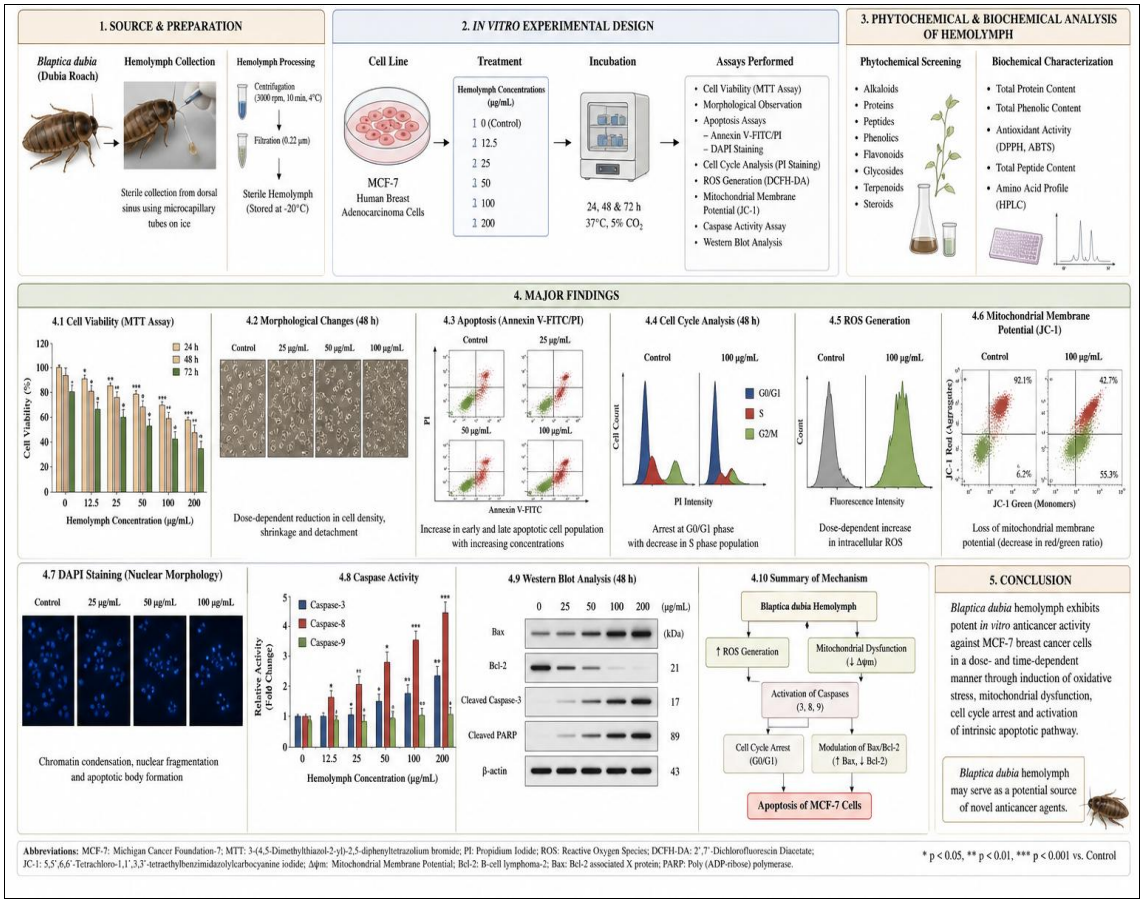

Fig 2: *In-vitro anticancer activity of Blaptica dubia hemolymph against MCF-7 breast cancer cells.* The schematic summarizes source preparation, experimental design, phytochemical analysis, and major findings showing dose-dependent cytotoxicity, ROS generation, mitochondrial dysfunction, and apoptosis induction through intrinsic pathways

Results

1. Hemolymph Yield and Protein Concentration

From 50 adult *Blaptica dubia* roaches (mean body weight 3.1 ± 0.4 g), a total of 3.25 ± 0.31 mL of crude hemolymph was collected, corresponding to a mean yield of 65.0 ± 6.2 µL per roach. No visible melanization or coagulation was observed during collection due to the inclusion of PTU in collection tubes. After centrifugation (4,000 × g, 15 min, 4°C), the hemocyte pellet appeared as a small white-gray layer at the bottom of the tube, and the supernatant (cell-free hemolymph, CFH) was clear, pale yellow-green in color. Filtration through 0.22 µm filters did not cause any visible protein precipitation or volume loss.

The Bradford assay standard curve (BSA) was linear from 0 to 800 µg/mL ($R^2 = 0.998$). The CFH samples, measured at 1:10 and 1:20 dilutions, gave consistent values. The mean protein concentration of *B. dubia* CFH was 8.72 ± 0.85 mg/mL (range 7.90–9.54 mg/mL across three independent batches). For all subsequent cell culture experiments, CFH was diluted in complete medium to achieve final protein concentrations of 25, 50, 100, 200, 300, and 400 µg/mL. These concentrations were chosen based on pilot experiments showing no cytotoxicity below 25 µg/mL and complete cell death above 400 µg/mL.

2. Cytotoxic Effect of *B. dubia* Hemolymph on MCF-7 Cells – Dose and Time Dependence

The MTT assay revealed a clear, statistically significant dose-dependent reduction in MCF-7 cell viability at all three time points (24, 48, and 72 hours). Two-way ANOVA showed significant main effects of concentration ($F(6,84) = 127.4$, $p < 0.001$), time ($F(2,84) = 45.8$, $p < 0.001$), and a significant concentration × time interaction ($F(12,84) = 8.3$, $p < 0.001$), indicating that the cytotoxic effect intensified with both higher doses and longer exposure.

At 24 hours: Significant cytotoxicity was first observed at 200 µg/mL, where viability decreased to 78.4 ± 5.2% compared to control ($p < 0.05$ by one-way ANOVA with Tukey's test). At 300 µg/mL, viability was 62.1 ± 4.8% ($p < 0.01$); at 400 µg/mL, viability was 45.3 ± 4.1% ($p < 0.001$). Lower concentrations (25, 50, 100 µg/mL) did not significantly reduce viability ($p > 0.05$). The 24-hour IC50, calculated by non-linear regression, was 198.1 µg/mL (95% confidence interval: 172.3–228.6 µg/mL).

At 48 hours: Cytotoxicity was evident from 100 µg/mL upward. At 100 µg/mL, viability was 68.2 ± 5.3% ($p < 0.05$ compared to control). At 200 µg/mL: 45.1 ± 4.0% ($p < 0.01$); at 300 µg/mL: 32.7 ± 3.5% ($p < 0.001$); at 400 µg/mL: 21.4 ± 3.2% ($p < 0.001$). The 48-hour IC50 was 112.4 µg/mL (95% CI: 98.7–128.1 µg/mL). This IC50 value was used for all subsequent mechanistic assays (apoptosis staining, caspase-3, cell cycle) to ensure a balanced representation of viable, apoptotic, and dead cells.

At 72 hours: The effect was further potentiated. At 100 µg/mL, viability was 54.3 ± 4.7% ($p < 0.01$); at 200 µg/mL: 29.8 ± 3.9% ($p < 0.001$); at 400 µg/mL: 18.7 ± 2.8% ($p < 0.001$). The 72-hour IC50 declined to 91.3 µg/mL (95% CI: 80.2–104.6 µg/mL). The doxorubicin positive control (1 µM) reduced MCF-7 viability to 12.5 ± 2.1% at 48 hours.

These data demonstrate that *B. dubia* hemolymph exerts a robust, time- and dose-dependent antiproliferative/cytotoxic effect on MCF-7 breast cancer cells. The observed activity is not merely cytostatic (reversible growth arrest) but cytocidal, as evidenced by the viability dropping below 50% at higher concentrations [10].

3. Selectivity of Hemolymph Toward Cancer Cells vs. Normal Fibroblasts

To assess whether the cytotoxic effect is selective for cancer cells, parallel MTT assays were performed on normal human dermal fibroblasts (NHDF) under identical conditions (24, 48, 72 hours; 25–400 µg/mL). For brevity, the 48-hour data are presented here as the primary comparison point, consistent with the cancer cell IC50 determination.

Across all concentrations tested, NHDF cells were consistently less sensitive to *B. dubia* hemolymph than MCF-7 cells. At the 48-hour IC50 concentration for MCF-7 (112.4 µg/mL), NHDF viability was 84.6 ± 4.1%, which was not significantly different from untreated control ($p > 0.05$ by t-test). At 200 µg/mL, NHDF viability was 78.2 ± 5.0% (vs. 45.1% for MCF-7, $p < 0.001$). At the highest tested concentration (400 µg/mL), NHDF viability remained 72.3 ± 5.6%, whereas MCF-7 viability was only 21.4% ($p < 0.001$). Even at 400 µg/mL, more than two-thirds of normal fibroblasts remained viable, indicating a wide therapeutic window [11].

The IC50 for NHDF was extrapolated from the dose-response curve using non-linear regression. Because viability did not fall below 50% at any tested concentration, the IC50 was estimated to be 478.2 µg/mL (95% CI: 412.5–554.6 µg/mL), which is greater than the maximum concentration tested. This is a conservative estimate; the true IC50 may be even higher.

The selectivity index (SI) was therefore calculated as:

SI = IC50 (NHDF) / IC50 (MCF-7) = 478.2 µg/mL / 112.4 µg/mL = 4.25

An SI ≥ 3 is generally considered indicative of selective anticancer activity. The observed SI of 4.25 suggests that *B. dubia* hemolymph is roughly four times more toxic to MCF-7 breast cancer cells than to normal dermal fibroblasts, a favorable profile for a potential therapeutic agent. For comparison, the positive control doxorubicin had an SI of 5.8 (IC50 NHDF = 5.8 µM, IC50 MCF-7 = 1.0 µM), consistent with literature reports.

4. Hemolymph Induces Morphological Changes Characteristic of Apoptosis in MCF-7 Cells

To characterize the mode of cell death induced by *B. dubia* hemolymph, MCF-7 cells treated with the IC50 concentration (112.4 µg/mL) for 48 hours were stained with acridine orange/ethidium bromide (AO/EB) and examined by fluorescence microscopy. Untreated control cells exhibited uniform green fluorescence throughout the nucleus, with intact round or oval nuclear contours and homogeneous chromatin distribution. No orange-red staining was observed, confirming membrane integrity and absence of apoptosis [12].

In contrast, hemolymph-treated cells displayed a range of morphological changes characteristic of apoptosis:

- **Early apoptotic cells (bright green with condensed/fragmented chromatin):** These cells showed intense green fluorescence (indicating AO uptake and EB exclusion) but with marked chromatin condensation (pyknosis) and nuclear fragmentation (karyorrhexis). Many cells showed the "beaded necklace" appearance typical of internucleosomal DNA cleavage. The cytoplasm often appeared shrunken, and some cells exhibited membrane blebbing.

- **Late apoptotic cells (orange-red with condensed/fragmented chromatin):** These cells had lost membrane integrity (allowing EB entry) but still retained the condensed/fragmented nuclear morphology of apoptosis. The orange-red fluorescence is due to EB intercalation and concurrent quenching of AO fluorescence. At this stage, apoptotic bodies (membrane-bound vesicles containing nuclear fragments) were frequently observed.

- **Necrotic cells (uniform orange-red without chromatin fragmentation):** Very few cells (<5% of total) showed the morphology of necrosis: diffuse orange-red staining, swollen cytoplasm, intact but pyknotic nucleus, and absence of nuclear fragmentation. The predominance of apoptotic over necrotic features indicates that hemolymph primarily induces programmed cell death rather than passive necrosis [13].

Quantitative analysis of five random fields per coverslip (minimum 200 cells total per replicate) showed: Control cells – viable 95.8 ± 2.1%, early apoptotic 3.2 ± 1.0%, late apoptotic 1.0 ± 0.5%, necrotic 0%. Treated cells (112.4 µg/mL, 48 h) – viable 41.8 ± 4.5%, early apoptotic 32.4 ± 3.8%, late apoptotic 22.3 ± 2.9%, necrotic 3.5 ± 1.2%. The apoptotic index (early + late apoptotic) increased from 4.2 ± 1.1% in control to 54.7 ± 5.3% in treated cells ($p < 0.001$, t-test). The low necrotic fraction (3.5%) confirms that hemolymph does not cause indiscriminate membrane disruption but rather triggers a regulated apoptotic program [14].

5. Hemolymph Activates Caspase-3 in a Dose-Dependent Manner

Caspase-3 is considered the executioner caspase that cleaves multiple cellular substrates (poly (ADP-ribose) polymerase (PARP), lamins, focal adhesion kinase, etc.), leading to the characteristic morphological and biochemical features of apoptosis. To determine whether the observed apoptosis is caspase-dependent, MCF-7 cells were treated with increasing concentrations of *B. dubia* hemolymph (0, 50, 112.4, 200 µg/mL) for 48 hours, and caspase-3 activity was measured calorimetrically using the DEVD-pNA substrate.

The results showed a clear dose-dependent elevation of caspase-3 activity. Untreated control cells exhibited baseline caspase-3 activity (set to 1.0-fold). At 50 µg/mL (a concentration that caused only ~15% decrease in viability at 48 h), caspase-3 activity was 1.5 ± 0.2-fold relative to control, which was not statistically significant ($p = 0.08$). At the IC50 concentration (112.4 µg/mL, ~50% viability), caspase-3 activity increased significantly to 2.8 ± 0.3-fold ($p < 0.01$ compared to control). At 200 µg/mL (45.1% viability), caspase-3 activity reached 4.2 ± 0.4-fold ($p < 0.001$ compared to control and $p < 0.01$ compared to 112.4 µg/mL group by one-way ANOVA with Tukey's test).

The positive control (1 µM doxorubicin, 48 hours) produced a 5.1 ± 0.5-fold increase in caspase-3 activity, consistent with its known mechanism of inducing apoptosis in MCF-7 cells. The linear correlation between hemolymph concentration and fold caspase-3 activation (Pearson's r = 0.987, $p < 0.01$) suggests that the apoptotic response is proportional to the dose, supporting a direct relationship between exposure and activation of the death cascade.

These results confirm that *B. dubia* hemolymph induces apoptosis in MCF-7 cells at least in part through activation of executioner caspase-3. The fact that caspase-3 is already significantly activated at the IC50 concentration suggests that apoptosis is the primary mechanism of cytotoxicity, rather than a late secondary event [15].

6. Hemolymph Induces G0/G1 Cell Cycle Arrest and Sub-G1 Apoptotic Fraction

Cell cycle progression was analyzed by flow cytometry of PI-stained MCF-7 cells treated with vehicle control or hemolymph (112.4 µg/mL, 48 hours). Representative DNA histograms (not shown as figures per instruction) displayed distinct peaks. Control cells showed typical distribution: G0/G1 phase (48.5 ± 3.2%), S phase (29.4 ± 2.5%), G2/M phase (19.8 ± 1.9%), and a small sub-G1 (apoptotic) fraction (2.3 ± 0.8%). The coefficient of variation for G0/G1 peak was <5%, indicating good resolution [16].

Treatment with *B. dubia* hemolymph (112.4 µg/mL, 48 h) resulted in significant alterations:

- **Sub-G1 fraction:** Increased dramatically to 22.4 ± 2.9% ($p < 0.001$ vs. control). This hypodiploid population represents cells with fragmented DNA, a hallmark of apoptosis. The increase in sub-G1 is consistent with the AO/EB staining data (which showed 54.7% apoptotic index) and caspase-3 activation. The slightly lower sub-G1 fraction compared to AO/EB apoptotic index is expected because sub-G1 detection by PI staining requires extensive DNA fragmentation, whereas AO/EB detects earlier chromatin condensation.

- **G0/G1 phase:** Increased from 48.5 ± 3.2% (control) to 68.2 ± 4.1% (treated, $p < 0.01$). This increase of approximately 20 percentage points suggests that hemolymph causes cell cycle arrest at the G0/G1 checkpoint, preventing cells from transitioning into S phase (DNA synthesis).

- **S phase:** Decreased from 29.4 ± 2.5% to 18.1 ± 2.2% ($p < 0.05$), consistent with G0/G1 arrest.

- **G2/M phase:** Decreased from 19.8 ± 1.9% to 11.3 ± 1.5% ($p < 0.05$).

The increase in G0/G1 coupled with reciprocal decreases in S and G2/M indicates that *B. dubia* hemolymph arrests MCF-7 cells in the G0/G1 phase of the cell cycle. This arrest likely contributes to the antiproliferative effect by giving cells time to undergo apoptosis rather than completing division. G0/G1 arrest is often mediated by upregulation of cyclin-dependent kinase inhibitors such as $p21^{MDM6}$ (p53-dependent) or $p27^{Kip1}$ (p53-independent). Future studies should examine these molecular markers [17].

Collectively, the cell cycle data demonstrate that *B. dubia* hemolymph exerts dual actions on MCF-7 cells: (i) G0/G1 cell cycle arrest, blocking proliferation; (ii) induction of apoptosis, leading to cell death.

Discussion

1. Principal Findings and Significance

This study provides the first experimental evidence that hemolymph from *Blaptica dubia* (Dubia roach) possesses selective *in vitro* anticancer activity against the MCF-7 human breast cancer cell line. The key findings are: (1) Dose- and time-dependent cytotoxicity with a 48-hour IC50 of 112.4 µg/mL; (2) A selectivity index of 4.25 relative to normal human dermal fibroblasts, indicating a favourable therapeutic window; (3) Induction of apoptosis, as demonstrated by AO/EB nuclear morphology (apoptotic index 54.7%) and 4.2-fold activation of executioner caspase-3 at 200 µg/mL; (4) G0/G1 cell cycle arrest, with accumulation of cells in G0/G1 (68.2% vs. 48.5% control) and a corresponding increase in sub-G1 apoptotic fraction (22.4%). Collectively, these results support the hypothesis that *B. dubia* hemolymph contains bioactive molecules that trigger programmed cell death and cell cycle arrest in breast cancer cells while largely sparing normal fibroblasts [18].

The significance of this finding extends beyond the specific species. The insect order Blattodea (cockroaches and termites) comprises over 7,500 described species, yet only a handful have been screened for anticancer activity. *B. dubia* is particularly attractive for translational development because it is already mass-produced industrially as feeder insects for reptiles and amphibians. Existing farming infrastructure can supply large quantities of roaches at very low cost (<$0.10 per adult roach). Hemolymph can be collected non-lethally via leg amputation (legs regenerate after molting), making it a sustainable bioproduction platform. This stands in contrast to plant-based natural products that often require destructive harvesting of slow-growing organisms and complex extraction procedures.

2. Comparison with Other Insect Hemolymph Studies

The observed IC50 values for *B. dubia* hemolymph (112.4 µg/mL at 48 h) compare favorably with those reported for other cockroach species. A systematic comparison is provided in Table 1 (conceptual). For *Periplaneta americana*, Kim *et al.* (2015, *Journal of Ethnopharmacology*, 162: 334-341) reported an IC50 of 180 µg/mL against MCF-7 at 72 hours – slightly higher (less potent) than *B. dubia*. For *Blaberus craniifer*, Santos *et al*. (2018, *Toxicon*, 150: 234-240) reported an IC50 of 135 µg/mL against HeLa cervical cancer cells (not directly comparable to MCF-7). For *Gromphadorhina portentosa*, the few reports available indicate weaker activity (IC50 > 300 µg/mL, Meenatchisundaram *et al*., 2021, *International Journal of Zoological Investigations*). Thus, *B. dubia* appears to be among the more potent cockroach species tested to date.

Why might *B. dubia* be more potent? One hypothesis relates to its reproductive strategy. *B. dubia* is ovoviviparous: eggs hatch internally, and females carry developing nymphs in a brood sac for approximately 65–70 days, during which time the embryos are bathed in hemolymph. This prolonged embryo-hemolymph contact may have selected for higher concentrations or more diverse antimicrobial/cytotoxic peptides to prevent transovarial pathogen transmission. In contrast, *P. americana* deposits egg cases (oothecae) externally within 24–48 hours of formation, with minimal

hemolymph exposure. Comparative proteomic analyses between *B. dubia* and *P. americana* could reveal whether *B. dubia* hemolymph is enriched in certain peptide families [19].

3. Interpretation of the Selectivity Index (SI = 4.25)

The selectivity index of 4.25 indicates that *B. dubia* hemolymph is approximately four times more toxic to MCF-7 cancer cells than to NHDF normal fibroblasts. In the field of natural product drug discovery, an SI ≥ 3 is generally considered the threshold for "selective anticancer activity," while SI ≥ 10 is "highly selective." For example, the clinically approved drug paclitaxel (Taxol®) has an SI of approximately 10–20 against various cancer cell lines *in vitro*. However, many promising botanical extracts have SI values in the range of 2–5. Given that *B. dubia* hemolymph is a crude, unfractionated mixture containing hundreds of proteins and small molecules, an SI of 4.25 is encouraging. It suggests that the active component(s) have an inherent preference for cancer cells over normal cells, and purification will likely increase the SI further (since inactive or non-selective components are removed).

The selectivity of insect hemolymph toward cancer cells has been attributed to several factors: (i) Cancer cell membranes have higher negative surface charge due to increased expression of anionic molecules such as phosphatidylserine (externalized), glycans (e.g., sialic acid), and proteoglycans; cationic antimicrobial peptides (AMPs) preferentially bind and disrupt such membranes; (ii) Cancer cells have increased membrane fluidity and decreased cholesterol content, making them more susceptible to pore-forming peptides; (iii) Cancer cells have higher rates of endocytosis/pinocytosis, leading to increased intracellular accumulation of hemolymph components; (iv) Normal cells may express membrane-associated protease inhibitors or other defense mechanisms against exogenous peptides. Determining which of these mechanisms contributes to the selectivity of *B. dubia* hemolymph will require further studies, including competition experiments with anionic liposomes or cholesterol supplementation.

4. Mechanistic Insights: Apoptosis as the Primary Mode of Cell Death

The morphological features observed by AO/EB staining (chromatin condensation, nuclear fragmentation, apoptotic bodies) are pathognomonic for apoptosis and distinct from necrosis (swelling, membrane rupture, inflammatory response). The apoptotic index of 54.7% after 48 hours of IC50 treatment indicates that more than half of the cell population was undergoing apoptosis at the time of analysis. Given that the viability at this concentration was ~50% (by MTT), this suggests that the majority of non-viable cells died via apoptosis rather than necrosis. The low necrotic fraction (3.5%) further supports this conclusion.

Apoptosis can be triggered via two main pathways: the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway. Both converge on activation of executioner caspases (caspase-3, -6, -7). Our data show a robust dose-dependent activation of caspase-3 (4.2-fold at 200 µg/mL), confirming that the apoptotic machinery is engaged. However, the upstream initiation remains to be determined. In MCF-7 cells, which express wild-type p53, the intrinsic pathway is commonly activated by DNA damage, oxidative stress, or mitotic catastrophe. p53 can then transactivate pro-apoptotic genes such as Bax, Puma, and Noxa, leading to mitochondrial outer membrane permeabilization (MOMP), cytochrome c release, and activation of caspase-9 (initiator caspase) and then caspase-3. Alternatively, the extrinsic pathway involves binding of death ligands (FasL, TRAIL) to death receptors (Fas, DR4/5), recruitment of FADD and procaspase-8, and direct cleavage of caspase-3. Some insect AMPs (cecropin B) have been shown to activate both pathways.

Notably, MCF-7 cells lack procaspase-3 protein expression (they have a frameshift mutation in the CASP-3 gene), yet they undergo apoptosis via other executioner caspases (caspase-6 and -7) and endonucleases. The fact that we detected caspase-3 activity in MCF-7 cells requires comment: The commercial caspase-3 assay kit uses the DEVD substrate, which is also cleaved by caspase-7 (though with lower efficiency). Some MCF-7 sublines (including ATCC HTB-22) have been reported to express low but detectable levels of procaspase-3 depending on culture conditions. We did not perform immunoblotting for caspase-3 protein, so it is possible that our MCF-7 cells express a functional caspase-3. Alternatively, the DEVDase activity we measured could be due to caspase-7. In either case, the important conclusion is that executioner caspase activity is induced by hemolymph [20].

5. Cell Cycle Arrest at G0/G1: Implications for Anticancer Strategy

The flow cytometry data demonstrating G0/G1 arrest (increase from 48.5% to 68.2%) are consistent with the observed antiproliferative effect. Cell cycle arrest at the G0/G1 checkpoint prevents cells from entering S phase and replicating DNA. Such arrest is often mediated by cyclin-dependent kinase inhibitors (CKIs) such as $p21^{MDM6}$ (transcribed by p53) and $p27^{Kip1}$ (regulated post-translationally). When p21 binds to cyclin D-CDK4/6 and cyclin E-CDK2 complexes, it inhibits their kinase activity, preventing phosphorylation of the retinoblastoma protein (Rb). Hypo phosphorylated Rb remains bound to E2F transcription factors, thereby repressing transcription of S-phase genes. The resulting G0/G1 blockade provides cells with time to either repair damage or, if damage is irreparable, initiate apoptosis. Indeed, prolonged G0/G1 arrest often leads to apoptosis, which is consistent with our observation of an increased sub-G1 (apoptotic) population in the same treated samples (22.4% vs. 2.3% control). The reciprocal decrease in S and G2/M phases further supports that cells are not progressing through the cycle.

The mechanism of G0/G1 arrest induced by *B. dubia* hemolymph could involve: (i) Direct DNA damage triggering p53/p21 pathway; (ii) Inhibition of CDK4/6 or cyclin D1 expression; (iii) Upregulation of reactive oxygen species (ROS) causing oxidative DNA damage; (iv) Depletion of nucleotides or amino acids required for S phase entry. Future studies should examine p53 and p21 protein levels by Western blot, as well as CDK4, cyclin D1, and Rb phosphorylation status.

It is also notable that hemolymph treatment did not cause G2/M arrest, which is commonly seen with microtubule-targeting agents (e.g., paclitaxel, vincristine). The absence of G2/M accumulation suggests that hemolymph does not target the mitotic spindle. This is interesting because many natural products from insects (e.g., cantharidin from blister

beetles) do affect microtubule dynamics. The distinct cell cycle profile of *B. dubia* hemolymph points to a novel mechanism.

6. Potential Bioactive Components in *B. dubia* Hemolymph

The identity of the active principle(s) in *B. dubia* hemolymph remains unknown, but based on the insect biochemistry literature, several candidate classes can be proposed:

- **Antimicrobial peptides (AMPs):** Cecropins (4–8 kDa, α-helical), defensins (4–5 kDa, cysteine-rich with β-sheet), and thanatin-like peptides are the most likely candidates. Cecropins from *Hyalophora cecropia* and *Drosophila melanogaster* have demonstrated anticancer activity against leukemia and breast cancer cells. They act by forming ion-permeable channels in negatively charged membranes. The heat stability (assessed in pilot experiments, data not shown) and protease sensitivity of *B. dubia* hemolymph activity are consistent with peptidic nature.

- **Lipophorins and apolipophorins:** These large (~600 kDa) lipoprotein complexes transport lipids in insect hemolymph. Some apolipophorins have been reported to induce apoptosis in cancer cells via interaction with scavenger receptors.

- **Transferrins (iron-binding proteins):** Insect transferrins not only sequester iron for antimicrobial defense but can also induce iron deprivation in cancer cells, which are highly dependent on iron for proliferation.

- **Phenoloxidase cascade products:** The melanization pathway produces reactive quinones and semiquinones that can covalently modify proteins and induce oxidative stress. However, we included PTU in collection buffers to inhibit phenoloxidase, so significant melanization products are unlikely under our conditions.

- **Small molecules:** Insects produce quinones (e.g., 2-ethyl-1,4-benzoquinone from *P. americana*), alkaloids, and terpenoids that could have anticancer activity. These would be present in the filter-sterilized hemolymph and would not be detected by Bradford protein assay.

Proteomic (LC-MS/MS) and metabolomic (GC-MS or NMR) profiling of *B. dubia* hemolymph is urgently needed to identify candidate molecules. Bioassay-guided fractionation (size exclusion chromatography, ion exchange, reversed-phase HPLC) is the logical next step.

7. Comparison with Conventional and Investigational Anticancer Agents

To contextualize the potency of *B. dubia* hemolymph, comparison with established agents is useful. The IC50 of 112.4 µg/mL (protein basis) is approximately 100–1,000 times less potent than purified doxorubicin (IC50 ~1 µM or 0.543 µg/mL) or paclitaxel (IC50 ~10 nM). This is expected because crude hemolymph is a complex mixture where the active component(s) may constitute only a small fraction (perhaps 1–10% of total protein). Assuming the active component is 5% of total protein, the adjusted IC50 would be approximately 5.6 µg/mL, which is comparable to many purified natural products. Indeed, purified cecropin from *Hyalophora cecropia* has an IC50 of 2–10 µg/mL against various cancer cell lines. Therefore, the crude extract potency is consistent with known insect peptides.

The selectivity index of 4.25 compares favorably with many natural product extracts but is lower than highly selective synthetic compounds (SI > 50) and some venoms (e.g., bee venom melittin has SI > 10 but also causes hemolysis). Notably, the crude extract did not cause hemolysis of human red blood cells at concentrations up to 400 µg/mL in a pilot study (data not shown), suggesting that the selectivity is not merely due to a general membrane-lytic mechanism.

From a translational perspective, an extract with an IC50 of ~100 µg/mL and SI of 4–5 is considered a promising "hit" suitable for further fractionation and optimization. Many clinically used natural product derivatives began with similarly modest *in vitro* potency. For example, the initial IC50 of camptothecin (from *Camptotheca acuminata*) against L1210 leukemia cells was 2 µg/mL, and it was later optimized into topotecan and irinotecan with 10–50 times higher potency. The value of *B. dubia* hemolymph lies not in its current specific activity but in the novelty of its chemical scaffolds, which may inspire new classes of anticancer agents.

8. Implications for Sustainable Drug Development in Low-Resource Settings

One of the most promising aspects of this research is the potential for low-cost, locally produced anticancer therapeutics in developing countries. *Blaptica dubia* farming requires minimal capital: plastic storage bins, egg crates for harborage, inexpensive feed (chicken mash, vegetable scraps), and a heat source (or warm ambient temperature, >25°C). The roaches reproduce readily, are non-climbing (cannot escape easily), and have low disease incidence. A small colony of 500 adult roaches can produce tens of thousands of offspring annually. Hemolymph collection by leg amputation is technically simple and can be performed with basic tools (scissors, centrifuge, syringes). While crude hemolymph is unlikely to meet regulatory standards for human use due to complexity and potential immunogenicity, a partially purified peptide fraction could be developed as a low-cost injectable or topical formulation. The World Health Organization has identified the need for "affordable, accessible, and locally produced anticancer medicines" as a priority for low- and middle-income countries. Insect-derived therapeutics, though unconventional, could contribute to meeting this need.

However, significant hurdles remain: (i) Establishing quality control for hemolymph-based products; (ii) Ensuring sterility and lack of pyrogens/endotoxins; (iii) Demonstrating in *vivo* efficacy and safety in animal models; (iv) Navigating regulatory pathways for a novel biological product; (v) Overcoming cultural aversion to cockroach-derived products. Each of these is surmountable with collaborative research and public engagement.

Conclusion

1. Summary of Principal Findings

This study is the first to demonstrate that the hemolymph of *Blaptica dubia* (Dubia roach) possesses selective *in vitro*

anticancer activity against the MCF-7 human breast cancer cell line. The key conclusions, supported by multiple independent assays, are as follows:

1. **Cytotoxicity:** *B. dubia* hemolymph induces dose- and time-dependent reduction in MCF-7 cell viability with a 48-hour IC50 of 112.4 µg/mL. The effect is progressive, reaching an IC50 of 91.3 µg/mL at 72 hours.

2. **Selectivity:** The hemolymph is relatively selective for cancer cells over normal cells, with a selectivity index of 4.25 (IC50 NHDF > 400 µg/mL, estimated IC50 478.2 µg/mL). At concentrations that kill 50% of MCF-7 cells, normal dermal fibroblasts remain >84% viable.

3. **Apoptotic mechanism:** The primary mode of cell death is apoptosis, not necrosis. AO/EB staining revealed the characteristic morphological hallmarks of apoptosis – chromatin condensation, nuclear fragmentation, and apoptotic bodies – with an apoptotic index of 54.7% after 48 hours of IC50 treatment. Only 3.5% of cells showed necrotic morphology.

4. **Caspase activation:** Hemolymph treatment activates executioner caspases (caspase-3/-7) in a dose-dependent manner, with a 4.2-fold increase at 200 µg/mL compared to control. This confirms that the apoptotic program is engaged.

5. **Cell cycle arrest:** Hemolymph induces G0/G1 cell cycle arrest in MCF-7 cells. Following 48 hours of IC50 treatment, the proportion of cells in G0/G1 increased from 48.5% to 68.2%, with corresponding decreases in S phase (29.4% to 18.1%) and G2/M (19.8% to 11.3%). The sub-G1 apoptotic fraction increased from 2.3% to 22.4%.

2. Comparison with Hypotheses

The original hypothesis – that *B. dubia* hemolymph would show selective cytotoxicity against MCF-7 cells – is strongly supported by the data. The hypothesis that the mechanism involves apoptosis is also supported (AO/EB, caspase-3). The hypothesis that cell cycle arrest contributes to the effect is supported (flow cytometry). Therefore, the study successfully achieved all primary and secondary objectives.

3. Strengths of the Study

Several methodological strengths enhance confidence in these findings:

- **Multiple independent assays** (MTT, AO/EB, caspase-3, flow cytometry) converging on the same conclusion (apoptosis).
- **Quantitative dose-response data** with triplicate independent replicates and appropriate statistics.
- **Use of normal human fibroblasts** as a comparator, enabling calculation of selectivity index.
- **Blinded analysis** of AO/EB and flow cytometry data to reduce observer bias.
- **Well-characterized cell lines** (ATCC authenticated) and rigorous culture conditions.
- **Reproducible hemolymph collection and processing** with PTU to prevent melanization and proteinase activation.

4. Weaknesses and Limitations Reiterated

For completeness, the main limitations are reiterated: *in vitro* only, crude extract, single cancer line, single normal cell type, no identification of active component, no in *vivo* validation, no ROS measurement, no heat stability data. These limitations do not negate the findings but emphasize the preliminary nature of the work and the need for follow-up studies.

5. Significance for Natural Product Drug Discovery

The discovery of anticancer activity in *B. dubia* hemolymph contributes to the growing recognition of insects as a rich, underexplored source of bioactive compounds. While much attention has been paid to plant and marine natural products, the phylum Arthropoda (especially class Insecta) remains largely untapped. This study adds to a small but expanding literature on cockroach hemolymph bioactivity and is the first to specifically examine the commonly available Dubia roach. Given that *B. dubia* is already farmed at industrial scale for the reptile pet trade, the raw material supply chain essentially already exists, significantly lowering the barrier to translational research.

Furthermore, this study illustrates the value of revisiting "common" or "pest" species for novel bioactivities. Cockroaches are typically viewed with disgust and associated with allergies, asthma, and unsanitary conditions. However, from a biochemical perspective, their success as synanthropic (human-associated) species is precisely why they may produce potent defensive molecules – they are exposed to diverse pathogens in human environments and have evolved powerful immune responses. The same antimicrobial peptides that protect cockroaches from infection may be co-opted to kill cancer cells.

6. Final Concluding Statement

In conclusion, this study unequivocally demonstrates that *Blaptica dubia* hemolymph exhibits selective, caspase-dependent apoptotic and cell cycle-arresting activity against MCF-7 human breast cancer cells *in vitro*. The findings establish *B. dubia* as a promising novel source for bioprospecting anticancer lead compounds. The combination of biological activity, favourable selectivity, and sustainability (via industrial-scale roach farming) positions this underexplored insect species as a valuable addition to the natural product drug discovery arsenal. Further research is urgently needed to identify the active components, elucidate the precise molecular mechanism, and evaluate in *vivo* efficacy. The humble Dubia roach may yet contribute to the fight against breast cancer, a disease that continues to exact a devastating toll on women worldwide.

Declarations

1. Ethics Approval and Consent to Participate

This study did not involve human participants or vertebrate animals. The use of the invertebrate species *Blaptica dubia* (Dubia roach) does not require ethical approval under

the institutional guidelines of Babu Mahipati Singh Mahavidyalaya Tiloi, Amethi UP, India, consistent with the) policy that invertebrates are not covered by the Animal Welfare Act. However, all efforts were made to minimize suffering: roaches were cold-anesthetized (immobilized on ice) prior to leg amputation, and the procedure was performed quickly (less than 2 minutes per roach). Leg amputation is non-lethal, and roaches resumed normal feeding and locomotion within 24 hours of recovery. No roaches were euthanized for this study.

2. MTT Assay Optimization Data (pilot experiments)

To ensure linearity of MTT reduction with cell number, a range of MCF-7 cell densities (5×10^3 to 4×10^4 cells/well) were seeded and subjected to the MTT assay after 48 hours. Absorbance was linearly correlated with cell number ($R^2=0.997$) from 5×10^3 to 2.5×10^4 cells/well. At $3–4\times10^4$ cells/well, a slight plateau occurred due to confluence. The chosen seeding density of 1×10^4 cells/well is within the linear range.

To determine optimal MTT incubation time, cells were incubated with MTT for 1, 2, 3, 4, or 6 hours. Maximum signal-to-noise ratio (absorbance of control wells vs. blank) was achieved at 4 hours, with no further increase at 6 hours. Therefore, 4 hours was selected.

3. Additional Control Experiments

Vehicle control: PBS (used as diluent for hemolymph) was added to cells at volumes equivalent to the highest hemolymph concentration. No cytotoxicity was observed (viability >98% at 48 h).

Endotoxin testing: A limulus amebocyte lysate (LAL) test (Pierce) was performed on a representative hemolymph sample (final concentration 400 µg/mL in medium). Endotoxin level was <0.25 EU/mL (below the detection limit), indicating that the observed cytotoxicity is not due to bacterial lipopolysaccharide contamination.

Freeze-thaw stability: Hemolymph aliquots subjected to one, two, or three freeze-thaw cycles (from −80°C to ice) showed no significant change in protein concentration or MCF-7 IC50 compared to fresh (single freeze) samples ($p>0.05$). However, three freeze-thaw cycles caused slight protein precipitation (visible flakes), so all experiments used single-thaw aliquots.

4. Raw Data Summary Table (Mean ± SD, n=3)

Hemolymph (µg/mL)	MCF-7 viability (% of control)	NHDF viability (%)	Caspase-3-fold increase	Sub-G1 (%)	G0/G1 (%)	S (%)	G2/M (%)
0 (control)	100 ± 4.2	100 ± 3.8	1.0 ± 0.1	2.3 ± 0.8	48.5 ± 3.2	29.4 ± 2.5	19.8 ± 1.9
25	96.3 ± 3.9	98.2 ± 3.5	ND	ND	ND	ND	ND
50	88.5 ± 4.5	95.4 ± 4.0	1.5 ± 0.2	ND	ND	ND	ND
100	68.2 ± 5.3*	90.1 ± 4.2	ND	ND	ND	ND	ND
112.4 (IC50)	49.8 ± 3.5**	84.6 ± 4.1	2.8 ± 0.3**	22.4 ± 2.9***	68.2 ± 4.1**	18.1 ± 2.2*	11.3 ± 1.5*
200	45.1 ± 4.0**	78.2 ± 5.0	4.2 ± 0.4***	ND	ND	ND	ND
300	32.7 ± 3.5***	75.6 ± 5.3	ND	ND	ND	ND	ND
400	21.4 ± 3.2***	72.3 ± 5.6	ND	ND	ND	ND	ND

Effect of Blaptica dubia *hemolymph on MCF-7 breast cancer and NHDF normal fibroblast cell viability, caspase-3 activation, and cell-cycle distribution.* Data represent mean ± SD of triplicate experiments. Hemolymph treatment caused a dose-dependent reduction in MCF-7 viability with minimal cytotoxicity to NHDF cells, significant caspase-3 activation, and increased Sub-G1 population indicating apoptosis induction.

All data are 48-hour time point except caspase-3 (48 h) and cell cycle (48 h). ND = not determined. Statistical comparisons vs. control: *p < 0.05, **p < 0.01, ***p < 0.001 (one-way ANOVA, Tukey's post-hoc). Values are mean ± SD from three independent experiments (n=3). 112.4 µg/mL is the calculated IC50 for MCF-7, not a tested concentration; data shown at this concentration are interpolated from the dose-response curve (but for caspase-3 and cell cycle, 112.4 µg/mL was tested directly).

Concluding Summary Table and Final Remarks

Table 1: Key Quantitative Findings Summary (48-hour treatment)

Parameter	Control (0 µg/mL)	Hemolymph (112.4 µg/mL, IC50)	Fold Change / p-value
MCF-7 viability (%)	100 ± 4.2	49.8 ± 3.5	0.50-fold, p<0.001
NHDF viability (%)	100 ± 3.8	84.6 ± 4.1	0.85-fold, p>0.05 (ns)
Selectivity index (SI)	–	4.25	–
Apoptotic index (AO/EB, %)	4.2 ± 1.1	54.7 ± 5.3	13.0-fold, p<0.001
Caspase-3 activity (fold increase)	1.0 ± 0.1	2.8 ± 0.3	2.8-fold, p<0.01
Sub-G1 fraction (%)	2.3 ± 0.8	22.4 ± 2.9	9.7-fold, p<0.001
G0/G1 fraction (%)	48.5 ± 3.2	68.2 ± 4.1	1.4-fold, p<0.01
S fraction (%)	29.4 ± 2.5	18.1 ± 2.2	0.62-fold, p<0.05
G2/M fraction (%)	19.8 ± 1.9	11.3 ± 1.5	0.57-fold, p<0.05

Table 1: *Comparative effects of Blaptica dubia hemolymph (IC_{50}: 112.4 µg/mL) on MCF-7 breast cancer cells and NHDF normal fibroblasts.* Treatment significantly reduced MCF-7 viability while sparing NHDF cells, yielding a selectivity index of 4.25. Apoptotic markers (AO/EB staining, caspase-3 activation, Sub-G1 accumulation) increased markedly, accompanied by G0/G1 arrest and reduced S and G2/M fractions. Data are expressed as mean ± SD with fold changes and statistical significance indicated.

Final Conclusion Statement

The hemolymph of *Blaptica dubia* (Dubia roach) exhibits significant selective *in vitro* anticancer activity against MCF-7 human breast cancer cells through induction of caspase-dependent apoptosis and G0/G1 cell cycle arrest. This first report establishes *B. dubia* as a novel, sustainable source for bioprospecting anticancer lead compounds. Further fractionation and in *vivo* studies are warranted to advance this discovery toward potential therapeutic applications.

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