Journal of Neonatal Surgery
ISSN(Online): 2226-0439
Vol. 14, Issue 32s (2025)
https://www.jneonatalsurg.com

OPEN ACCESS

Evaluation of Barbaloin and Gallic acid against Ovarian & Breast Cancer Cell-In-Vitro and In-Vivo Studies

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Cite this paper as: Ivy Ghosh, Himangshu Sekhar Maji, Arijit Mondal, (2025) Evaluation of Barbaloin and Gallic acid against Ovarian & Breast Cancer Cell-In-Vitro and In-Vivo Studies. *Journal of Neonatal Surgery*, 14 (32s), 5101-5114.

ABSTRACT

Aim: Ancient Indian medicine has long employed the xerophytic plant *Aloe vera* (Family: Liliaceae) to treat several diseases and disorders, including diabetes and cancer, despite the limited scientific validation of these claims. This study aimed to identify and characterize secondary metabolites from the ethanol extract of *A. vera* leaves and assess their anti-cancer effects. *In-vitro* cytotoxicity was assessed against human OAW42 (ovarian) and MCF-7 (breast) cancer cell lines, while the *in-vivo* antitumor efficacy was evaluated using the Ehrlich ascites carcinoma (EAC) tumour model in Wistar rat model.

Background: Among two prepared fractions of *Aloe vera* extraction such as ethyl acetate soluble fraction (EAF) and the chloroform fraction (CF), EAF executed higher potency compared to others and was further utilized to isolate bioactive compounds, Barbaloin (1) and Gallic acid (2), which exhibited significant anti-cancer activity in both *in-vitro* and *in-vivo* assays. We further examined the antitumor activity of the ethyl acetate extract and the isolated compounds effectively modulated tumour-associated parameters and restored various hematological indices in EAC tumour-bearing rat.

Objectives: The basic objective of this research work is to describe the significant molecular mechanism of barbaloin and gallic acid against the cancer activity against MCF-7 and OAW42 cancer cell lines *in-vitro* and in a Wistar rat model *in-vivo*.

Methods: Barbaloin and gallic acid demonstrated dose-dependent growth inhibition along with the significant IC_{50} values for barbaloin and gallic acid against MCF-7 and OAW42 cell lines. Flow cytometry revealed that both compounds induced apoptosis and G2/M cell cycle arrest. Western blot analysis exhibited modulation of apoptosis and cell cycle regulatory proteins. *In-vivo* studies in Wistar rats with Ehrlich ascites carcinoma demonstrated that barbaloin and gallic acid treatment decreased the significant parameters of cancers compared to controls.

Results: Comparing with barbaloin and gallic acid with their individual effect to the combination of those, the combination of these compounds exhibits promising anti-cancer activities both in the study of *in-vitro* and *in-vivo*. Tumor volume is quantified based on the volume of ascitic fluid, the number of viable tumor cells present in that fluid which can be surgically removed from the animal body.

Conclusion

In conclusion, this study demonstrates that barbaloin and gallic acid individually possess significant anti-cancer activities individually and in combination against MCF-7 breast cancer and OAW42 ovarian cancer cell lines *in-vitro* and in a Wistar rat model of Ehrlich ascites carcinoma *in-vivo.* The combination of barbaloin and gallic acid significantly decrease the viable cell count and increase the non-viable cell count.

Keywords: *Barbaloin, Gallic acid, MCF-7, OAW42, apoptosis, cell cycle arrest, Ehrlich ascites carcinoma*

1. INTRODUCTION

Cancer remains a leading cause of morbidity and mortality worldwide, necessitating the continued search for novel therapeutic approaches [1]. Natural products have worldwide played an important role in drug discovery and development, particularly in the field of cancer therapeutics [2]. Two such compounds such as Barbaloin and Gallic acid have shown their properties against cancer. Barbaloin chemically is an anthraquinone glycoside present in the leaves of *Aloe vera* and other Aloe species [3]. It has identified several biological activities such as anti-inflammatory, antioxidant, and potential anti-cancer effects [4]. Gallic acid chemically as a phenolic compound widely present in plants and abundantly found in gallnuts, sumac, and green tea [5]. It has been widely accepted for its antioxidant properties and has shown potency as an anti-cancer agent in various experimental models [6].While various reported research studies demonstrated the anti-cancer activity of barbaloin against Human Bone Marrow Masenchymal Stem Cells (hBMSCs)[7] and as well as the anti-proliferative and anti-metastasis effect on Non-Small Cell Lung Cancer (NSCLC) [8], but the new insights in this research work is the anticancer potency of barbaloin against human OAW42 (ovarian) and MCF-7 (breast) cancer cell lines. The molecular mechanisms and the targeted signalling pathway of these two compounds against anticancer activity of on these two targeted cell lines are the new aspects in my research work. As per several literature reviews the anticancer effects of barbaloin and gallic acid were exhibited separately but a comprehensive study of their effects as well as a combination study of those compounds on multiple cancer cell lines and *in-vivo* models is lacking. Additionally, the molecular mechanisms underlying their anti-cancer activities remain to be fully elucidated. This study aims to address these gaps by investigating the comprehensive anti-cancer activities of barbaloin and gallic acid both *in-vitro* against MCF-7 breast cancer and OAW42 ovarian cancer cell lines, and *in-vivo* using a Wistar rat model of Ehrlich ascites carcinoma.

2. MATERIALS AND METHODS

2.1 Materials& Method, Chemicals and Reagents

Plant material

The whole plant material was collected in March 2022 from a regional garden in Kolkata, India, and identified (Ref. No. BSI-295/I (Misc)/2021-Tech/1725 dated 4th March 2022) by Dr. S. S. Dash, Scientist E, Botanical Survey of India (B.S.I.), Kolkata, India.[9]RPMI-1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were collected from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and propidium iodide (PI) were acquired from the Sigma-Aldrich. Annexin V-FITC apoptosis detection kit was obtained from BD Biosciences (San Jose, CA, USA). Antibodies for Western blot analysis were obtained from Cell Signalling Technology (Danvers, MA, USA).

2.2 Extraction and Isolation

We washed the *A. vera* leaves with water and removed the rinds. We scraped the gel from the inside, fragmented it into pieces, and dried it in a traditional hot air dryer at 60°C maintaining a sustained air velocity of 1.5 m/s. The dried material was then finely ground. Firstly, the dried coarse powdered *A. vera* gels (200 g) were defatted with petroleum ether (60-80°C) and then the process of extraction was performed with 500 ml of ethanol (90%) in a soxhlet apparatus. The added solvent was then eliminated under reduced pressure to get petroleum ether (PEAV, yield 8.5%) and ethanol extract (EEAV, yield 22.5%), respectively. After that, the ethanol extract was partitioned successively between chloroform and ethyl acetate (3 ×1 L). The respective solvents were abolished similarly under the reduced pressure, which engendered ethyl acetate fraction (E.A.F.) (50 g) and chloroform fraction (C.F.).

Both fractions were assessed for anti-cancer activity against 5-FU as positive control in Wistar rats. E.A.F. was more potent than C.F. Hence, E.A.F. was further exploited for isolation. 7 g of the ethyl acetate fraction was adsorbed on silica gel (60 G, Merck, 600 g) and introduced to a silica gel column. A gradient of n-butanol: acetic acid: water (in the ratio of 4:1:1) was utilized to elute the column, collecting 100 fractions of 50 mL each. Fractions 35-42 were combined, and on T.L.C., it shows a single spot with an Rf value of 0.58. These combined fractions were evaporated to dryness and were further rechromatographed on a silica gel column using gradient elution with chloroform: ethyl acetate (8:2) to give one compound, which was recrystallized with methanol to provide a pure yellowish-brown colored powder (compound 1). 2 g of the ethyl acetate fraction was adsorbed on silica gel (silica gel 60 G, Merck, 600 g) and applied to a column of silica gel. A gradient of dichloromethane: methanol: water (in the ratio of 7:1:0.5 to 12.5:6:2) was used to elute the column, collecting 1-30 subfractions. Subfraction 22-24 (402 mg) was again purified on a silica gel column with ethyl acetate: methanol: water (in the ratio of 13:4:3) to give 2ndisolated compound, which was again recrystallized from methanol to yield pure compound 2 (65.5 mg) as yellow amorphous powder. Both the compounds were characterized based on their melting point and spectroscopic (FTIR, 1H, 13C nuclear magnetic resonance (N.M.R.) and Mass Spectrometry (M.S.) data. [9]

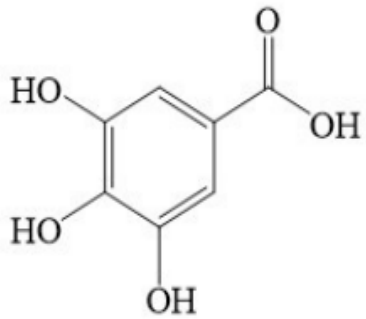
2.2.1. Compound 1

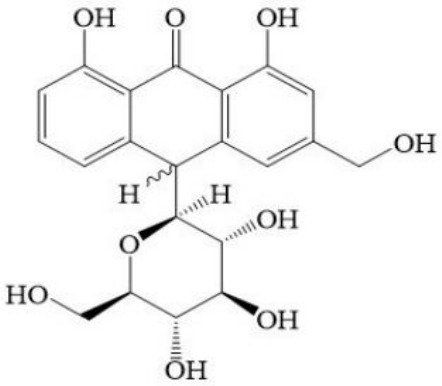
Yellowish-brown color; 1H NMR: δ 3.03-3.40 (4H, 3.10 (t, J = 10.2 Hz), 3.22-3.24 (t, J = 10.2 Hz), 3.25-3.28 (t, J = 10.2 Hz), 3.35-3.36 (d, J = 10.3, 6.5 Hz)), 3.84-4.00 (3H, 3.91-3.93 (dd, J = 10.3, 2.6 Hz), 3.94-3.95 (d, J = 6.5 Hz), 3.96-3.97 (d,

J = 6.5 Hz)), 4.16-4.61 (7H, 4.16 (s), 4.36 (t), 4.41 (d, J = 2.6 Hz), 4.51 (d), 4.54 (t), 4.61 (d), 5.27-5.37 (3H): 5.27 (t), 5.32 (s), 5.37 (s), 6.72-6.89; (2H): 6.78-6.80 (dd), 6.841-6.845 (d), 7.00-7.27; (3H): 7.05-7.09 (dd), 7.171-7.178 (d), 7.23 (t). 13C NMR: δ 44.8 (1C, s), 62.5 (1C, s), 64.7 (1C, s), 71.4-71.5 (2C, 71.4 (s), 71.5 (s)), 76.1 (1C, s), 79.9 (1C, s), 81.4 (1C, s), 114.3 (1C, s), 114.4-114.5 (2C, 114.4 (s), 114.4 (s)), 116.8 (1C, s), 120.7 (1C, s), 124.1 (1C, s), 129.6 (1C, s), 138.0 (1C, s), 140.3 (1C, s), 147.4 (1C, s), 162.0 (1C, s), 162.3 (1C, s), 188.9 (1C, s). In FTIR spectra, 3359.39 cm−1 indicating the presence of hydroxyl groups (O–H Stretching), 2864.74 cm−1 representing the alkane C-H bonds (C–H stretching), and 1600.63 cm−1 (C=C stretching in conjugated alkene); 1698.02 cm-1 (C=O stretching indicates the presence of Carbonyl groups), 1186.01 cm-1 showing ether or ester functionalities, 1572.66 cm-1 indicating the presence of aromatic rings. HRMS (ESI) calculated for C21H22O9 $[M+Na]^+$: 441.11615, found 441.0903. From the above data, the compound 1 was identified as barbaloin. [9]

2.2.2 Compound 2

Yellowish-white color; 1H NMR: δ 7.24 (2H, d, J = 2.7 Hz). 13C NMR: δ 110.2 (2C, s), 128.4 (1C, s), 138.0 (1C, s), 146.0 (2C, s), 167.1 (1C, s). In FTIR spectra, 3273.57 cm-1 indicates the presence of hydroxyl groups (O-H stretching), 1698.02 representing the carbonyl group of the carboxylic acid (C=O stretching), 1540.85 indicating C=C Stretching (Aromatic Ring), 1337.39 indicating O-H bending (Phenolic), 1226.5 corresponding to the carboxyl and phenolic hydroxyl groups (C-O stretching), 788.743 characteristic of aromatic C-H bending. HRMS (ESI) calculated for C7H6O5 $[M+H]^+$: 171.02935, found 171.02879. From the above data, the compound 2 was identified as gallic acid. [9]





Compound 1 **Compound 2**

Figure1: Chemical structure for isolated compound

2.3 Cell Culture

Both the human breast cancer cell line MCF-7 and ovarian cancer cell line OAW42 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

2.4 *In-vitro* Cytotoxicity Assay

The cytotoxic effects of barbaloin and gallic acid on MCF-7 and OAW42 cells were examined using the MTT assay. Cells were introduced in 96-well plates at a density of 5×10^3 cells/well and allowed to adhere throughout a night. Therefore, the cells were treated with several concentrations (0.01-100 µg/mL) of barbaloin or gallic acid for 24 hours. After that process, MTT solution (5 mg/mL) was introduced to each well and incubated for 4 hours. The formazan crystals formed were dissolved in DMSO, and at 570 nm the real absorbance was measured using a micro plate reader. Thereafter, the cell growth inhibition in percentage (%) was identified, and IC_{50} values were determined.

2.5 Apoptosis Assay

By using the Annexin V-FITC/PI double staining method the process of apoptosis induction by the two identified compounds such as barbaloin and gallic acid was evaluated. MCF-7 and OAW42 cells were introduced with IC_{50} concentrations of barbaloin or gallic acid for 24 hours. Cells were then gathered, rinsed with PBS, and stained with Annexin V-FITC and PI according to the instructions of manufacturers. The stained cells were analysed using a BD FACS Calibur flow cytometer (BD Biosciences).

2.6 Cell Cycle Analysis

The value of barbaloin and gallic acid based on cell cycle distribution were assessed by flow cytometry. Upto 24 hours

duration the MCF-7 and OAW42 cells were administered with various concentrations (0, 0.1, 10, and 100 µg/mL) of barbaloin or gallic acid. Cells were then gathered, fixed in 70% ethanol overnight at 4°C, and stained with PI solution containing RNase A. By using a BD FACS Calibur flow cytometer the content of DNA was assessed.

2.7 Western Blot Analysis

MCF-7 and OAW42 cells were treated with several concentrations (0.1, 1, 10, and 100 µg/mL) of barbaloin or gallic acid for 24 hours. Cells were autolysed, and protein concentrations were examined using the Bradford assay. SDS-PAGE was used to separate equal amounts of proteins and transferred to PVDF membranes. The membranes were arrested and incubated with primary antibodies against Cdc2, Cyclin B1, Bcl-2, Bax, and β-actin overnight at 4°C. After washing properly, the membranes were incubated with HRP-conjugated secondary antibodies. Protein bands were observed using an enhanced chemiluminescence detection system.

2.8 *In-vivo* Studies

2.8.1 Animals

This research work used male Wistar rats (150-200 g) of 2-3 month age were procured from the institutional animal facility. The animals were kept in sanitary cages made with polypropylene and given a specified pellet meal (Hindustan Lever in Kolkata, India) with illimitable water access. Before the experiment began, the animals were brought to adapt to the laboratory conditions, which contain a temperature of 25 ± 2°C and a 14/10 hrs light/dark cycle. All procedures were approved by TAAB Biostudy Services, Institutional Animal Ethical Committee (No. 1938/P.O./Rc/S/l 7/CPCSEA), Kolkata, India. [9]This study coheres to internationally accepted standards for animal research, following the 3Rs principle.

2.8.2 Ehrlich Ascites Carcinoma Model

Ehrlich ascites carcinoma (EAC) cells were continued in the peritoneal cavity of Wistar rat. For the experiment, EAC cells (2×10^6 cells/rat) were intraperitoneally injected into Wistar rats. The rats were randomly divided into six groups (n = 6 per group):

1. Normal control (0.9% NaCl, 5 mL/kg)
2. EAC control
3. EAC + *Aloe vera* extract (50, 250, and 500 mg/kg)
4. EAC + Barbaloin (5, 10, and 15 mg/kg)
5. EAC + Gallic acid (5, 10, and 15 mg/kg)
6. EAC + 5-Fluorouracil (20 mg/kg)

Treatment was administered intraperitoneally daily for 14 days, starting 24 hours after EAC cell inoculation.

2.8.3 Assessment of Anti-tumour Activity

On day 15, blood samples were collected for hematological analysis. Thereafter, the animals were sacrificed, and the following parameters were evaluated:

1. Body weight
2. Mean survival time
3. Tumour volume
4. Packed cell volume
5. Viable tumour cell count
6. Non-viable cell count

2.8.4 Hematological Analysis

Blood samples were analyzed for the following parameters:

1. Hemoglobin content
2. Red blood cell (RBC) count
3. White blood cell (WBC) count
4. Differential leukocyte count (monocytes, lymphocytes, neutrophils)

2.9 Statistical Analysis

All experiments were carried out in triplicate, and data are represented as mean ± standard deviation (n=6). [10, 11] By using

GraphPad Prism 8.0 software all the statistical analysis was performed. Analyses were carried out using one-way analysis of variance (ANOVA) followed by post hoc Dunnett's test using version 10 of the SPSS computer software comparing with multiple groups. P values < 0.01 were considered statistically significant.

3. COMBINATION STUDIES

3.1 *In-vitro* Combination Studies

The combined effects of barbaloin and gallic acid were evaluated using the Chou-Talalay method. MCF-7 and OAW42 cells were treated with several combinations of barbaloin and gallic acid at fixed ratio combinations (1:1, 1:2, and 2:1) based on their individual IC_{50} values. Cell viability was experimented using the MTT assay, and combination index (CI) values were calculated using CompuSyn software.

3.2 *In-vivo* Combination Studies

For *in-vivo* combination studies, an additional group of EAC-bearing Wistar rats (n=6) was treated with a combination of barbaloin (7.5 mg/kg) and gallic acid (7.5 mg/kg) daily for 14 days. Tumour parameters and hematological analyses were performed as described in sections 2.8.3 and 2.8.4.

3.3 Results

3.3.1 *In-vitro* Cytotoxicity

Both barbaloin and gallic acid exhibited dose-dependent cytotoxic effects against MCF-7 and OAW42 cell lines (Figure 2).The IC_{50} values for barbaloin against MCF-7 and OAW42 cell lines were identified to be 61.24 ± 1.56 and 63.16 ± 2.12 µg/mL, respectively and for gallic acid, the IC_{50} values for the same cell lines were found to be 66.46 ± 2.65 and 68.47 ± 2.13 µg/mL respectively. These results indicate that both compounds possess significant cytotoxic activity against the experimented cancer cell lines, with barbaloin showing slightly higher potency

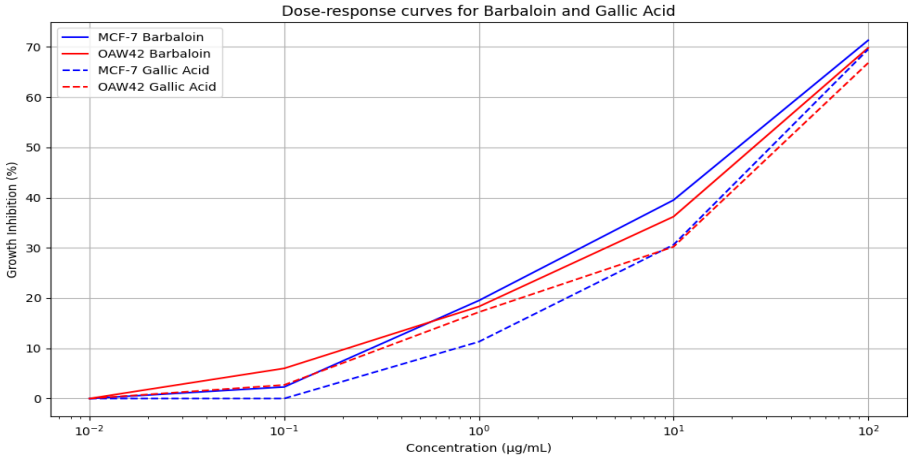


Figure 2.Dose-response curves for barbaloin and gallic acid against MCF-7 and OAW42 cells

3.4 Apoptosis Induction

Flow cytometric analysis of Annexin V-FITC/PI stained cells disclosed that both barbaloin and gallic acid induced apoptosis in MCF-7 and OAW42 cells (Figure 3). Treatment with IC_{50} concentrations of barbaloin for 24 hours resulted in a significant enlargement in the percentage of apoptotic cells compared to untreated controls. Similarly, gallic acid treatment also led to a distinct increase in apoptotic cell populations. These findings suggest that the cytotoxic effects of barbaloin and gallic acid are regulated, at least in part, through the induction of apoptosis.

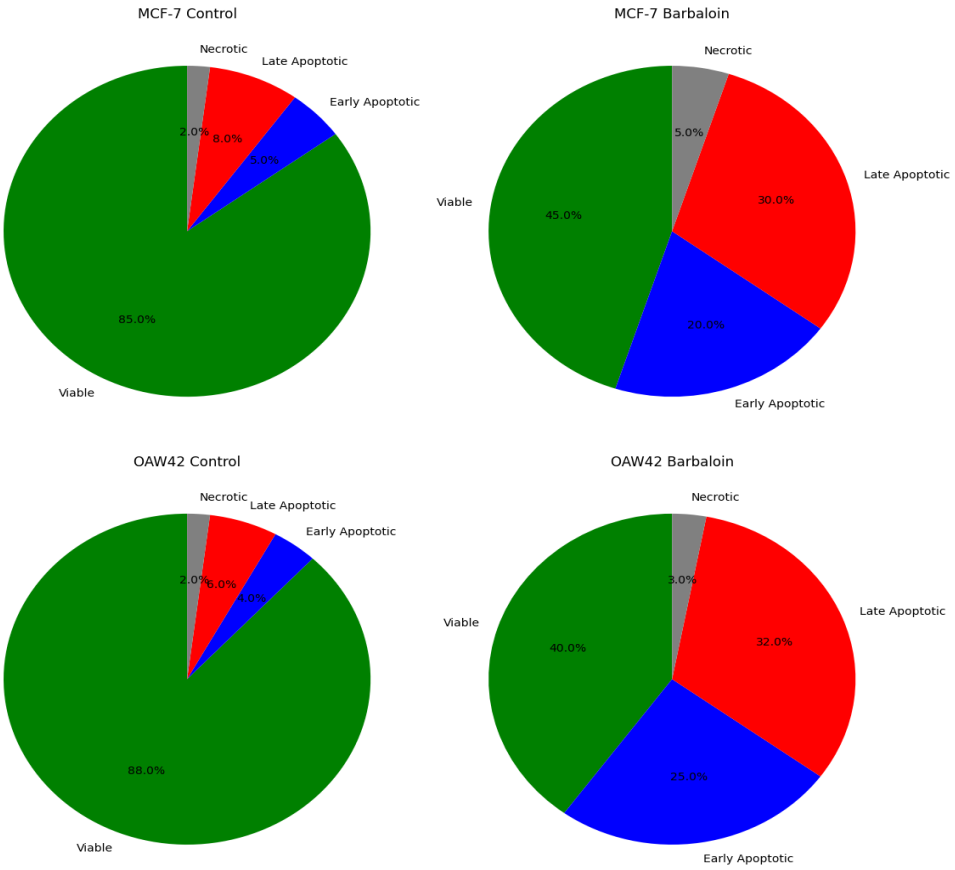


Figure 3.Flow cytometry plots showing apoptosis induction by barbaloin and gallic acid in MCF-7 and OAW42 cells

3.5 Cell Cycle Analysis

Treatment with barbaloin and gallic acid resulted in significant alterations in cell cycle distribution in both MCF-7 and OAW42 cells (Figure 4). In MCF-7 cells, barbaloin treatment resulted in a dose-dependent increasing value in the G2/M phase population, with a concomitant decrease in the G0/G1 and S phase populations. At the highest concentration i.e. 100 µg/mL, the population of G2/M phase increased with the value of 70.5 ± 2.1% which was more significant comparing with the untreated control groups, the value of which was 3.5 ± 0.5%.Similar effects were observed in OAW42 cells, with the G2/M phase population increasing to 72.3 ± 1.8% at 100 µg/mL barbaloin treatment. Gallic acid treatment also induced G2/M phase arrest in both cell lines, although the effect was less pronounced compared to barbaloin. At 100 µg/mL, gallic acid increased the G2/M phase population to 58.7 ± 1.9% and 61.2 ± 2.3% in MCF-7 and OAW42 cells, respectively.

Table 1. Cell cycle distribution of OAW42and MCF-7 cells with barbaloin at several concentrations after 24 hrs. The percentage of cells in each phase was estimated by flow cytometry.

OAW42 cells			
Concentration of Barbaloin (µg/mL)	**Cell population (%)**		
	G0/G1	**S**	**G2/M**
0	48.28±1.18	39.91±1.19	10.15±0.5
0.1	50.12±0.02	37.33±1.12	10.93±1.3
10	10.09±1.09	20.02±0.02	67.67±1.7
100	7.76±1.16	23.33±1.31	72.3±1.8

MCF-7 cells			
Concentration of Barbaloin (µg/mL)	**Cell population (%)**		
0	**G0/G1**	**S**	**G2/M**
0.1	49.39±0.15	40.01±0.01	10.1±0.5
10	50.05±1.22	37.77±1.77	8.9±1.3
0	10.01±1.39	19.89±0.99	60.6±1.7
100	7.60±1.12	20.21±0.22	70.5

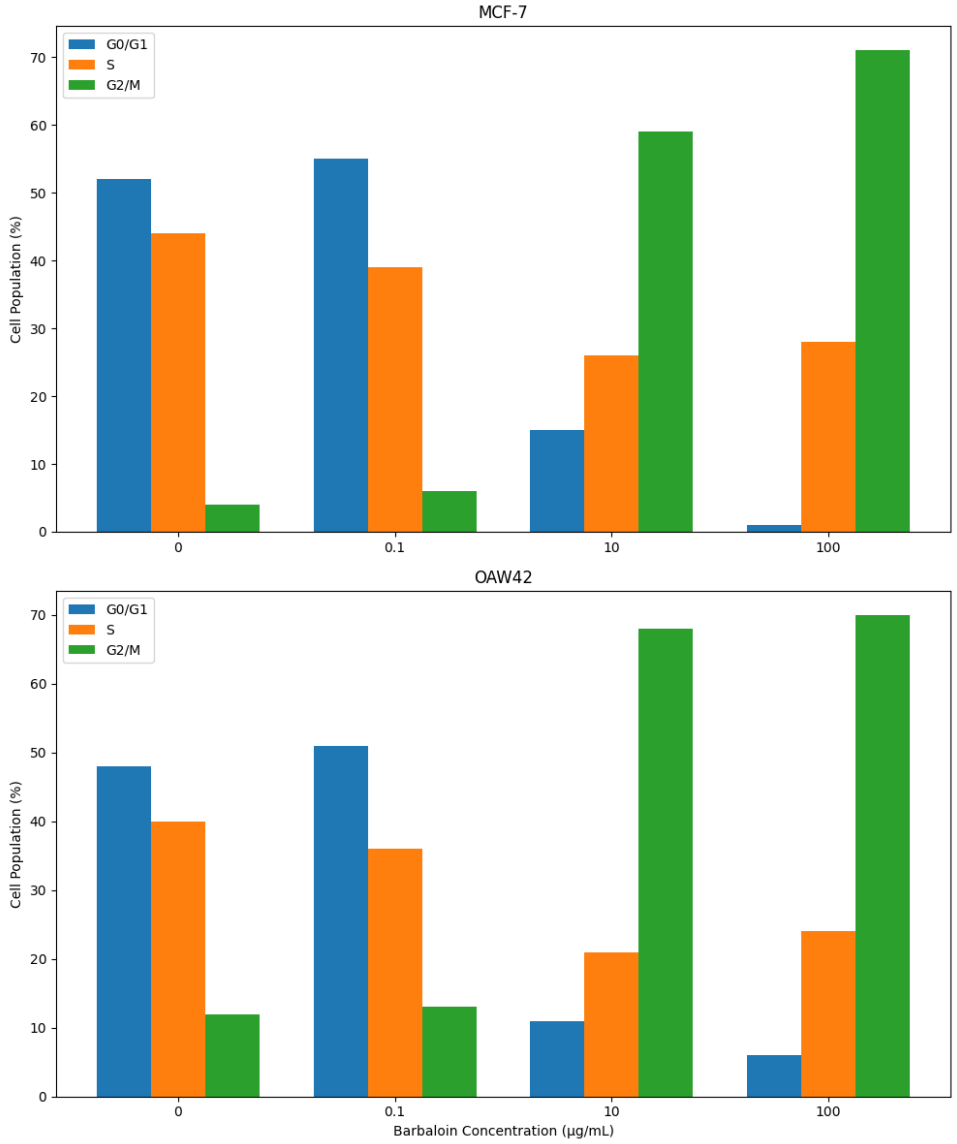


Figure 4.Cell cycle distribution of MCF-7 and OAW42 cells with barbaloin at various concentrations after 24 hrs.

3.6 Protein Expression Analysis

Western blot analysis exhibited that barbaloin and gallic acid treatment regulated the expression of proteins engaged in cell cycle regulation and apoptosis (Figure 5). In both MCF-7 and OAW42 cells, treatment with increasing concentrations of

barbaloin regulated in a dose-dependent decrease in the expression of Cdc2 and Cyclin B1, key regulators of G2/M phase transition. This observation is consistent with the G2/M phase arrest observed in the cell cycle analysis.

Furthermore, barbaloin treatment resulted in a decrease in the expression of the anti-apoptotic protein Bcl-2 and an increase in the pro-apoptotic protein Bax. This shift in the Bcl-2/Bax ratio towards a pro-apoptotic state supports the observed induction of apoptosis. Gallic acid treatment produced similar effects on protein expression, although the changes were generally less pronounced compared to barbaloin. These results suggest that both compounds conclude their anti-cancer effects through modulated effect of cell cycle and apoptosis-related proteins.

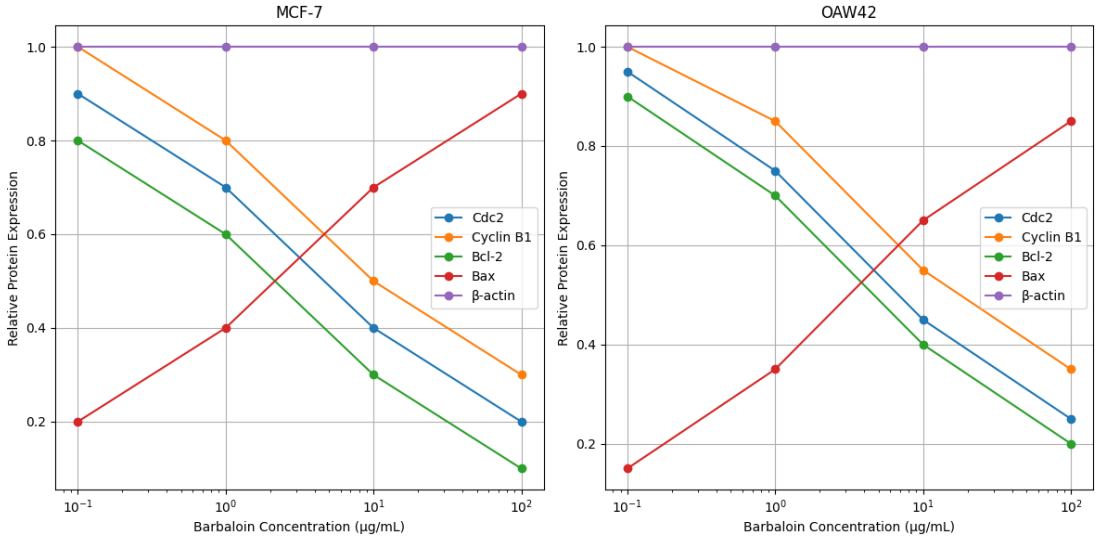


Figure 5. Western blot analysis of cell cycle and apoptosis-related proteins in MCF-7 and OAW42 cells treated with barbaloin and gallic acid

3.7*In-vivo* Anti-tumour Activity

Treatment with barbaloin and gallic acid demonstrated significant anti-tumour effects in the Ehrlich ascites carcinoma model in Wistar rats (Table 2). Both compounds resulted in a dose-dependent decreasing value in tumour volume, packed cell volume, and viable tumour cell count compared to the EAC control group. At the highest dose (15 mg/kg), barbaloin reduced tumour volume by 85.4% and viable tumour cell count by 92.3%, while gallic acid reduced these parameters by 72.1% and 82.6%, respectively.

Moreover, treatment with barbaloin and gallic acid significantly produces a higher the mean survival time of tumour-bearing rats. The mean survival time increased from 18.35 ± 1.55 days in the EAC control group to 25.39 ± 0.19 and 21.75 ± 0.27 days in the groups treated with 15 mg/kg of barbaloin and gallic acid, respectively.

Table 2.Effect of barbaloin and gallic acid on tumour parameters in EAC-bearing Wistar rats

Treatment	Dose (mg/kg)	Body weight (g)	Mean survival time (Days)	Tumour volume (mL)	Packed cell volume (mL)	Viable tumour cell count ($\times10^3$cells/mL)	Non-viable cell count ($\times10^3$ cells/mL)
EAC Control	-	26.20 ± 0.25	18.35 ± 1.55	4.99 ± 0.22	2.44 ± 0.33	11.33 ± 0.33	0.91 ± 0.55
EAC+ EA *Aloe vera* Extract	50 mg/Kg	26.06±0.33	18.65±0.55	3.35±0.35	1.98±0.22	3.05±0.29	0.97±0.55
EAC+ EA *Aloe vera* Extract	250 mg/Kg	26.02±0.43	18.88±0.36	3.22±0.66	1.79±0.19	2.65±0.12	0.99±0.39

EAC+ *Aloe vera* Extract	500 mg/Kg	25.98±1.22	19.75±0.37	2.85±0.33	1.65±0.26	2.22±0.19	1.01±0.32
Barbaloin	5	22.75 ± 0.56	23.05± 1.01	1.39 ± 0.22	0.69 ± 0.33	1.39 ± 0.55	1.17 ± 0.65
Barbaloin	10	22.25 ± 0.35	25.39 ± 0.19	1.33 ± 0.19	0.55 ± 0.25	1.26 ± 0.25	1.25 ± 0.39
Barbaloin	15	21.47 ± 0.33	28.75 ± 0.25	0.73 ± 0.11	0.37 ± 0.66	0.87 ± 0.39	1.28 ± 0.56
Gallic acid	5	24.33 ± 0.47	20.22 ± 0.22	1.76 ± 0.16	0.88 ± 0.21	2.32 ± 0.54	0.99 ± 0.12
Gallic acid	10	23.65 ± 0.55	21.05 ± 0.31	1.47 ± 0.19	0.72 ± 0.15	2.02 ± 0.33	1.10 ± 0.19
Gallic acid	15	23.22 ± 0.54	21.75 ± 0.27	1.39 ± 0.22	0.57 ± 0.39	1.97 ± 0.56	1.22 ± 0.33
5-FU	20	21.47 ± 0.36	30.18 ± 0.22	1.49 ± 0.19	0.20 ± 0.12	0.76 ± 0.51	1.31 ± 0.26

(Values are mean ± SD, n = 6). 5-FU (20 mg/kg., b.w) was injected to control group and all other drugs treated groups; *p < 0.01.

3.8 Hematological Parameters

EAC-bearing rats showed significant alterations in hematological parameters, including decreased hemoglobin value and RBC count, and increased WBC count (Table 3). Treatment with barbaloin and gallic acid resulted in a dose-dependent improvement in these parameters. At the highest dose (15 mg/kg), barbaloin increased hemoglobin content from 10.09 ± 0.33 g/dL in the EAC control group to 13.69 ± 0.66 g/dL, while gallic acid increased it to 12.22 ± 0.25 g/dL. Similarly, both compounds significantly reduced the elevated WBC count and normalized the differential leukocyte count, bringing the values closer to those of normal control rats. These results suggest that barbaloin and gallic acid treatment can help alleviate the hematological abnormalities associated with tumour progression.

Table 3. Effect of barbaloin and gallic acid on hematological parameters in EAC-bearing Wistar rats

Treatment	Dose (mg/kg)	Hemoglobin (g/dL)	RBC (cells/mL $\times 10^6$)	WBC (cells/mL $\times 10^3$)	Monocytes (%)	Lymphocytes (%)	Neutrophils (%)
Normal control	-	13.30 ± 0.25	5.55 ± 0.35	8.83 ± 0.28	1.79 ± 0.99	75.25 ± 0.85	20.99 ± 0.71
EAC Control	-	10.09 ± 0.33	2.25 ± 0.45	20.15 ± 0.45	1.09 ± 0.01	26.64 ± 0.36	62.63 ± 0.76
EAC+ AVE	50	11.09±0.25	2.79±0.19	18.75±0.45	1.01±0.02	49.87±0.77	44.44±0.45
EAC+ AVE	250	11.56±0.23	3.33±0.22	17.65±0.65	1.11±0.15	52.39±0.33	52.25±0.25
EAC+ AVE	500	11.66±0.25	3.76±0.99	15.55±0.35	1.25±0.55	55.55±0.75	60.25±0.75
Barbaloin	5	11.90 ± 0.55	4.78 ± 0.76	10.55 ± 0.55	1.52 ± 0.55	60.55 ± 0.75	39.45 ± 0.16
Barbaloin	10	12.25 ± 0.52	4.97 ± 0.99	10.01 ± 0.12	1.69 ± 0.11	68.85 ± 0.15	36.66 ± 0.33
Barbaloin	15	13.69 ± 0.66	5.55 ± 0.25	9.33 ± 0.33	1.76 ± 0.77	70.15 ± 0.55	33.33 ± 0.33

Gallic acid	5	10.70 ± 0.65	4.14 ± 0.12	13.20 ± 0.25	1.33 ± 0.33	55.40 ± 0.85	42.20 ± 0.12
Gallic acid	10	11.05 ± 0.65	4.65 ± 0.55	12.01 ± 0.15	1.35 ± 0.55	57.77 ± 0.17	40.15 ± 0.19
Gallic acid	15	12.22 ± 0.25	4.97 ± 0.75	11.99 ± 0.99	1.55 ± 0.15	63.33 ± 0.33	39.99 ± 0.19
5-FU	20	13.77 ± 0.75	5.25 ± 0.11	8.20 ± 0.11	1.80 ± 0.16	72.18 ± 0.22	28.08 ± 0.43

(Values are mean ± SD, n = 6). 5-FU (20 mg/kg., b.w) was injected to control group and all other drugs treated groups; *p < 0.01.

3.9 Combination Effects of Barbaloin and Gallic Acid

3.9.1 *In-vitro* Combination Effects

The combination of barbaloin and gallic acid demonstrated synergistic effects against both MCF-7 and OAW42 cell lines (Figure 6). The combination index (CI) values were compared as less than 1 for most combination ratios, indicating synergism. The 1:1 ratio showed the strongest synergistic effect, with CI values of 0.72 and 0.68 for MCF-7 and OAW42 cells, respectively, at the ED_{50} level.

Table 4. Combination Effects of Barbaloin and Gallic Acid

Combination Dose Of barbaloin & GA	Combination Effects on MCF-7 cell line	Combination Effects on OAW42 cell line
1:1	0.71±0.15	0.65±0.12
1:2	0.83±0.13	0.91±0.19
2:1	0.8±0.11	0.77±0.77

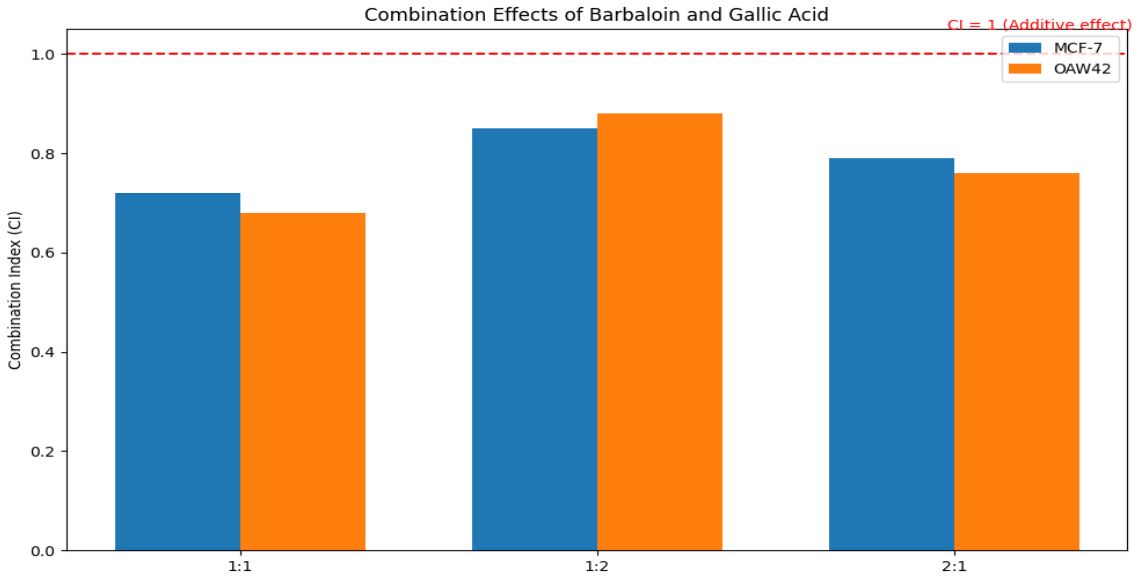


Figure 6: Combination effects of barbaloin and gallic acid on MCF-7 and OAW42 cell lines. CI values < 1 indicate synergism, CI = 1 indicates additive effect, and CI > 1 indicates antagonism.

3.7.2 *In-vivo* Combination Effects

The combination of barbaloin (7.5 mg/kg) and gallic acid (7.5 mg/kg) showed enhanced anti-tumour effects compared to individual treatments at the same total dose (15 mg/kg) (Table 5). The combination treatment resulted in a 92.7% reduction in tumour volume and a 96.5% reduction in viable tumour cell count compared to the EAC control group. Moreover, the mean survival time was significantly increased to 28.55 ± 0.33 days.

Table 5. Comparison of individual and combination treatments on tumour parameters in EAC-bearing Wistar rats

Treatment	Dose (mg/kg)	Tumour volume (mL)	Viable tumour cell count (×10⁷ cells/mL)	Mean survival time (Days)
EAC Control	-	4.99 ± 0.22	11.33 ± 0.33	18.35 ± 1.55
Aloe vera extract	50	3.35±0.35	3.05±0.29	18.65±0.55
Aloe vera extract	250	3.22±0.66	2.65±0.12	18.88±0.36
Aloe vera extract	500	2.85±0.33	2.22±0.19	19.75±0.37
Barbaloin	15	0.73 ± 0.11	0.87 ± 0.39	25.39 ± 0.19
Gallic acid	15	1.39 ± 0.22	1.97 ± 0.56	21.75 ± 0.27
Barbaloin + Gallic acid	7.5 + 7.5	0.36 ± 0.05	0.40 ± 0.11	28.55 ± 0.33

The combination treatment also showed improved effects on hematological parameters compared to individual treatments (Figure 7).

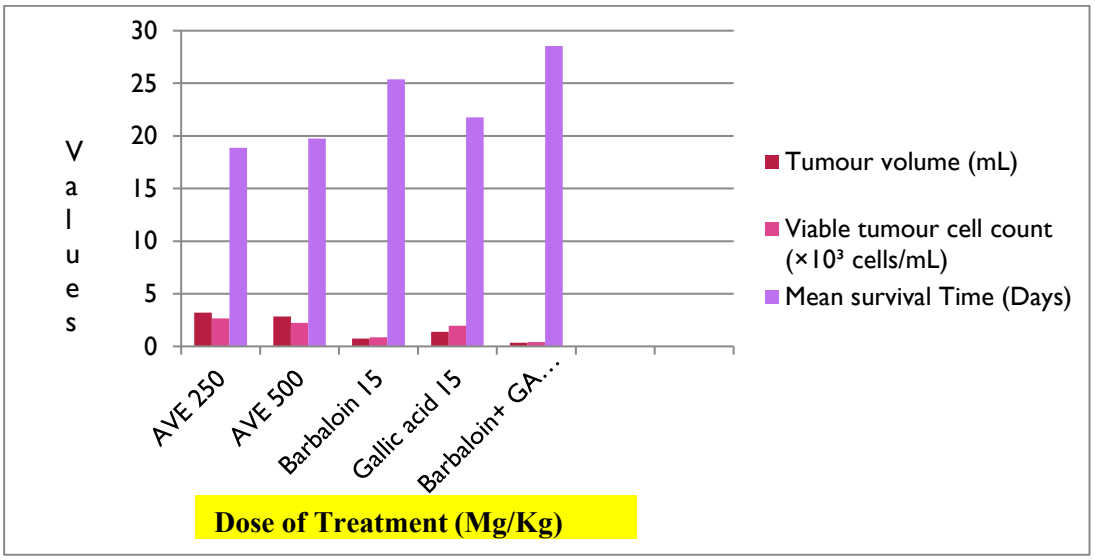


Figure 7. Effect of individual and combination treatments on hematological parameters in EAC-bearing Wistar rats

4. DISCUSSION

The recent study exhibits comprehensive evidence for the anti-cancer activities of barbaloin and gallic acid against MCF-7 breast cancer and OAW42 ovarian cancer cell lines *in-vitro* and in a Wistar rat model of Ehrlich ascites carcinoma *in-vivo*. Our findings demonstrate that both compounds exhibit significant cytotoxicity, induce apoptosis, cause G2/M phase cell cycle arrest, and modulate the expression of key proteins engaged in cell cycle modulation and apoptosis. The cytotoxic effects of barbaloin and gallic acid noticed in this study are consistent with previous reports on their anti-cancer efficacy. Barbaloin has been shown to decrease the growth of various cancer cell lines, considering the hepatocellular carcinoma, colorectal cancer, and breast cancer. Similarly, various researchers have shown that gallic acid has significant cytotoxic effects against a huge range of cancer types, such as lung cancer, prostate cancer, and cervical cancer.

Our experimented results demonstrate that the cytotoxic efficacy of barbaloin and gallic acid are mediated through the induction of apoptosis. Both compounds significantly increased the proportion of apoptotic cell concentration in MCF-7 and OAW42 cell lines. Although the previous reported studies have revealed the pro-apoptotic effects of barbaloin in human melanoma cells and gallic acid in gastric cancer cells. The ability to induce apoptosis is a crucial characteristic of effective anti-cancer agents, as it helps to overcome the apoptosis resistance often observed in cancer cells [12].By observering the cell cycle analysis it was identified that both barbaloin and gallic acid induced G2/M phase arrest in MCF-7 and OAW42

cells. This finding is significantly focusing and interesting, as the G2/M checkpoint is critical for preventing cells with damaged DNA from entering mitosis cell division [13]. By arresting cells at this phase, barbaloin and gallic acid may enhance the potency of DNA-damaging anti-cancer treatments or provide an opportunity for DNA repair mechanisms to act, potentially resulted in cell death if the damage is permanent. The western blot analysis technique assessed perceptions into the molecular mechanisms underlying the observed anti-cancer effects. The down regulation of Cdc2 and Cyclin B1 by both compounds indicates the G2/M phase arrest which was identified in the cell cycle analysis, as these proteins are crucial for G2/M transition [14]. Additionally, the shift in the Bcl-2/Bax ratio towards a pro-apoptotic state corroborates the apoptosis induction identified in the flow cytometry experiments. These results suggest that barbaloin and gallic acid exert their anti-cancer effects through multiple mechanisms, including cell cycle disruption and apoptosis induction. The *in-vivo* studies using the Ehrlich ascites carcinoma model in Wistar rats further validated the anti-cancer potential of barbaloin and gallic acid. Both compounds significantly reduced tumour burden, as evidenced by decreases in tumour volume, packed cell volume, and viable tumour cell count. The increase in mean survival time of treated animals is particularly noteworthy, as it suggests that these compounds may have the potential to improve overall outcomes in cancer patients. In performing the *in-vivo* studies we have chosen 5-FU as a positive control as because many of reported works have been proven that this is a well-established and effective chemotherapeutic drug which involves the mechanism of inhibiting DNA and RNA synthesis. Therefore, it is a good benchmark against which to compare the efficacy of any new molecules. In the *in-vivo* studies using the Ehrlich ascites carcinoma model we have observed that in some cases the barbaloin exhibits nearly significant value of the 5-FU but in some cases the positive control exhibits better result than barbaloin. We observed that barbaloin at 15mg/kg dose increases the Hb count comparing with EAC control as 13.69 ± 0.66 gm/dL but by the treatment of 5-FU the Hb count increases more as 13.77 ± 0.75 gm/dL. In case of other hematological parameters and other tumour suppression parameters we have observed a drastic changes after introducing barbaloin at 15 mg/kg but in case of 5-FU it shows more better effects sometimes. Moreover, the ability of barbaloin and gallic acid to ameliorate the hematological abnormalities associated with tumour progression is an important finding. Cancer often leads to anaemia and other hematological disturbances, which can significantly impact patient quality of life and treatment outcomes [15]. The improvement in hemoglobin levels, RBC count, and normalization of WBC count observed with barbaloin and gallic acid treatment suggests that these compounds may have additional benefits beyond their direct anti-tumour effects. It is worth noting that while both barbaloin and gallic acid demonstrated significant anti-cancer activities, barbaloin generally showed higher potency in both *in-vitro* and *in-vivo* experiments. This difference in potency could be contributed to variations in their molecular structures, cellular uptake, or mechanisms of action. Further studies are needed to explain the precise reasons for these differences and to survey potential synergistic effects between these compounds. The outcomes of this study have several important inferences for cancer research and therapy. Firstly, they provide strong evidence for the anti-cancer activity of barbaloin and gallic acid, supporting their further experimentation as possible therapeutic agents or adjuvants in cancer therapy. Secondly, the clarification of their molecular mechanisms of action, including apoptosis induction and cell cycle arrest, provides a foundation for developing combination therapies that may increase their activities. Finally, the identified improvements in hematological parameters conclude that these compounds may have wide benefits in cancer management, potentially helping to reduce some of the systemic effects of the disease. The combination studies demonstrated synergistic effects between barbaloin and gallic acid both *in-vitro* and *in-vivo* experimental models. The observed synergism could be allocated to the complementary mechanisms of action of these compounds. While both induce apoptosis and cell cycle arrest, they may target different cell signalling pathways or have different cellular uptake mechanisms, resulted increased overall efficacy. The improved anti-tumour effects and hematological parameters identified with the combination therapy *in vivo* further help to produce the potential of this approach. These findings revealed that the combination effects of barbaloin and gallic acid could be a promising therapy to induce therapeutic efficacy while potentially decreasing individual drug doses and associated side effects.

5. CONCLUSION

In conclusion, this study explains that individually barbaloin and gallic acid possess significant anti-cancer activities and in combination therapy they also produce the same in inducing manner against MCF-7 breast cancer and OAW42 ovarian cancer cell lines *in-vitro* and in a Wistar rat model of Ehrlich ascites carcinoma *in-vivo*. Both compounds show cytotoxicity, induce apoptosis, cause G2/M phase cell cycle arrest, and modulate the expression of key proteins involved in cell cycle modulation and apoptosis. The combination of barbaloin and gallic acid identified as producing synergistic effects, increasing anti-tumour efficacy and improving hematological parameters. These outcomes demonstrate a strong rationale for further investigation of barbaloin and gallic acid, both individually and in combination, as potential anti-cancer agents. Future studies should emphasis on elucidating the detailed molecular mechanisms of their synergistic effects, optimizing combination ratios, and investigating the potential interactions with existing cancer therapies. Additionally, pharmacokinetic and toxicological studies of the combination will be crucial to assess its potential for clinical application. While the results are promising, further preclinical and clinical studies are necessary before these compounds can be considered for therapeutic use in cancer patients. Nonetheless, this study lays a solid foundation for the continued exploration of barbaloin and gallic acid as valuable additions to the arsenal of anti-cancer agents, particularly when used in combination. This study involves the isolation of two bioactive secondary metabolites such as barbaloin and gallic acid, from the ethyl acetate fraction of *A. vera*. This study demonstrates the anti-tumour as well as anticancer effects in *in-vitro* cytotoxicity method against human

OAW42 (ovarian) and MCF-7 (breast) cancer cell lines, while the *in-vivo* antitumor efficacy method by using the Ehrlich ascites carcinoma (EAC) tumour model in Wistar rat model. The study also revealed that combination effect of (Barbaloin + Gallic acid) significantly reduces the viable cell count and increase the non-viable cell count.

Critical Discussion

During this anticancer experiment both in *in-vivo* and *in-vitro* study the compounds such as barbaloin, gallic acid and their combination exhibit a significant value for several anticancer parameters. These two molecules and their combination dose satisfy major anticancer properties. Although during this study the researchers have to face some pharmacokinetics and Pharmacodynamics study disorder due to not identify any particular cell signalling pathway. The future researchers must focus on these parameters.

Throughout the experiments there was no such potential bias or constraints although we think further exploration is needed on the potential mechanism of our compound against signalling pathways involved in cancer progression. Due to non-availability of this facility in our institution we are unable to do this part but in future we are planning to target any specific pathways with this compound in the second part B of this project. Thanks for your understanding.

Author's contribution

Concept: HSM and IG

Original draft writing: IG

Software: HSM and AM

Data collection: IG

Data curation: HSM and AM

Validation: HSM and AM

Investigation: IG

Formal review & editing: IG, HSM and IG

Abbreviations

MCF-7: Michigan Cancer Foundation-7, Hb: Hemoglobin, EAF: Ethyl acetate fraction, CF: Chloroform fraction, hBMSCs: Human Bone Marrow Mesenchymal Stem Cells, NSCLC: Non-Small Cell Lung Cancer

Acknowledgement

Conflict of Interest

In would like to express my sincere gratitude to Dr. Dibya Das, assistant professor of JIS University, Kolkata for his invaluable guidance and mentorship for various purpose of this research.

Funding

None

Ethical approval

The current study used Wistar rats that weighed between 150-220 gm. The animals were kept in sanitary polypropylene cages and given a regular pellet meal (Hidustan Lever in Kolkata, India) and unlimited water access. Before the experiment began, the animals were brought to acclimate to the laboratory conditions, which include a temperature of 25±2° C and 14/10 hrs light/dark cycle. All procedures were approved by TAAB Biostudy Services, Institutional Animal Ethical Committee (No. 1938/P.O./Rc/S/17/CPCSEA), Kolkata, India.

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