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RESEARCH ARTICLE

Exploring the Antitumour Potential of *Diospyros chloroxylon* Roxb. Extract in EAC Models: An Integrative *In Vitro* and *In Vivo* Approach

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Abstract

Background: Natural compounds have emerged as promising alternatives, owing to their low toxicity and potential efficacy. This study investigates the anticancer effects of *Diospyros chloroxylon* Roxb. leaf extract on Ehrlich Ascites Carcinoma (EAC) *in vitro* and *in vivo*. **Methods:** The *in vitro* cytotoxicity of *D. chloroxylon* extract was assessed using the MTT assay on various cancer cell lines, including EAC, A549 (lung), MCF-7 (breast), DU 145 (prostate), HT 29 (colon) and Human Umbilical Vein Endothelial Cells (HUVECs). The *in vivo* study involved the treatment of EAC-bearing mice with two doses (200mg/kg and 400mg/kg). Parameters such as body weight, tumour volume, packed cell volume, viable and non-viable cell counts, mean survival time and lifespan were evaluated. Haematological parameters and biochemical markers were also analysed, followed by histopathological analysis. **Results:** In the MTT assay, *D. chloroxylon* extract showed selective cytotoxicity, exhibiting a strong effect on EAC cells with lower IC_{50} values than other cancer cell lines and minimal toxicity towards HUVECs. In *in vivo*, *D. chloroxylon* treatment mitigated weight loss, reduced tumour volume in a dose-dependent manner and improved survival times. It also normalised haematological and biochemical parameters, indicating its potential to manage cancer-induced complications. Histopathological studies showed that doxorubicin and higher doses of *D. chloroxylon* enhanced liver tissue structure. However, complete recovery from EAC-induced hepatic alterations, such as dilated sinusoids, remains elusive. **Conclusion:** *Diospyros chloroxylon* Roxb. leaf extracts demonstrated significant anticancer activity *in vitro* and *in vivo*. Its ability to selectively induce cytotoxic effects on cancer cells and its beneficial effects in an EAC mouse model suggests its potential as a therapeutic agent for cancer treatment.

Keywords: Antitumour, *Diospyros chloroxylon,* EAC Model, MTT Assay

1. Introduction

The genus Diospyros consists of over 700 species of shrubs and trees, mostly found in tropical regions, with some species native to temperate climates. Known as persimmons or ebony trees, these plants vary widely. Some are valued for their dense, black wood, while others are cultivated for their fruits. The species *Diospyros chloroxylon* Roxb. or *Ullintha*, is native to the Indian subcontinent and grows as a small deciduous tree. It is notable for its dark brown bark, yellowish-grey wood used in crafting tools and musical instruments and its ecological role in dry deciduous forests of India. The tree bears small, bell-shaped flowers and produces a berry-like fruit, enjoyed by local tribes. It has also been used in traditional *Siddha* medicine to treat various ailments[1-4].

Several species in this genus have yielded phytochemical components that have been isolated, including terpenoids, ursanes, lupanes, polyphenols, tannins, hydrocarbons and lipids as well as benzopyrones, naphthoquinones, oleananes and taraxeranes. 5(-)-Isodiospyrin, a novel binaphthoquinone and diospyrin were isolated from *D. chloroxylon* wood. 7-methyl juglone and xylospyrin, a novel trinaphthylenequinone, were isolated from *D.*

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chloroxylon[5-7]. The leaves have been reported to have a wide array of secondary metabolites such as cardiac glycosides, alkaloids and tannins[8,9].

Cancer is a severe disease that is characterised by unchecked cell growth and tumour formation and presents a significant threat to global health. It causes organ damage and metastasis through a complicated interaction of variables, including genetics, environment, and lifestyle. Despite major research efforts, developing anticancer medicines that are both effective and have minimal adverse effects is still challenging. Cancer is the world's most significant cause of death, accounting for approximately 10 million deaths in 2020, primarily affecting the elderly. The most frequent malignancies are lung, breast, colon and rectal cancers, with lung cancer being the most common. Socioeconomic concerns about this trend are becoming more widespread in rich and developing countries[10,11].

On the other hand, natural medications, mostly made from herbs are becoming increasingly popular as viable substitutes because of their cultural acceptability, safety and low side effects, especially when used consistently. Modern drug development includes cutting-edge methods for identifying active plant components with a growing emphasis on natural sources. The increasing acceptance of alternative therapies, which are renowned for their high patient tolerance and minimal toxicity, is driving this shift. As a result, complementary or alternative natural medicines are increasingly being used in place of conventional treatments, providing new hope in the fight against cancer and other disorders[12,13].

The potential of this plant species as a rich source of naturally occurring chemicals with anticancer activities needs to be determined through research. Its pharmacological effects require thorough study to develop new natural medicinal medicines. This research could lead to the discovery of novel anticancer medications with fewer side effects than conventional chemotherapeutic medications.

2. Materials and Methods

2.1 Plant Materials

Diospyros chloroxylon leaves were collected from Hyderabad, Telangana in November 2022 and authenticated from the Botanical Survey of India, Hyderabad (BSI/DRC/2023-24/Identification/98).

2.2 Reagents and Chemicals

All the chemicals and reagents were procured from Sigma Aldrich (laboratory grade).

2.3 Preparation of Extracts

Aerial parts of *D. chloroxylon* leaves were harvested and dried in the shade. Dried leaves were powdered and defatted using n-hexane, followed by ethanolic extraction (70% ethanol) through maceration. The resulting extracts were filtered and the solvent was removed by rotary evaporation to yield a solid extract. The yield percentage was calculated and recorded[14].

2.4 Phytochemical Screening

Preliminary phytochemical analysis of *D. chloroxylon* extracts was performed according to the established protocols[15].

2.5 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide (MTT) Assay

MTT assay was used to determine the cytotoxic potential of *D. chloroxylon* A 549 (lung), DU 145 (prostate), HT 29 (colon), MCF-7 (breast) cancer cell lines and Primary HUVECs as a control were obtained from the American Type Culture Collection (ATCC). These cell lines were incubated with test samples in a 96-well plate under standard conditions (37°C, 5% CO_2, 72 hours). After treatment with MTT salt (20µl, 2mg/ml, phosphate-buffered saline) and subsequent incubation for 3 hours under the same conditions, the purple formazan produced by the reduction of MTT salt by mitochondrial enzymes was extracted with DMSO (100µl). The intensity of the coloured formazan was measured in triplicate using a spectrophotometer (540 nm) and the intensity of the formazan represented the viability of the cells. This intensity was proportional to the number of living cells and was expressed as IC_{50} values. The obtained values were compared with the standard (Doxorubicin) and blank[16].

2.6 Acute Oral Toxicity Studies

Swiss albino mice were used as the animal model following the Organisation for Economic Co-operation and Development (OECD) guidelines 423. A limited test dose of 2000mg/kg body weight was administered for the experiment. Before initiating

each experimental run, all the animals were subjected to overnight fasting, although unrestricted access to water was provided. Animals were divided into five groups of six mice each. The first group functioned as a negative control, while the second through fifth groups were administered varying dosages of the plant extracts, specifically 5, 50, 300, and 2000mg/kg of body weight, via oral gavage[17].

The body weight of each animal was recorded before the administration of the test compounds to ensure accurate dosing. Initial observations for potential toxicological effects were conducted during the four-hour window following extract administration. Subsequent monitoring was extended over three days, during which a range of physiological and behavioural parameters, including body weight, urinary output, food and water consumption, respiratory rate, presence of convulsions or tremors, gastrointestinal motility and ocular and dermal pigmentation were closely examined.

The Bhaskar Medical College Animal Ethics Committee (CCSEA Registration number 1758/PO/Re/S/14/CCSEA) thoroughly examined and granted approval for all experimental procedures.

2.7 Ehrlich Ascites Carcinoma (EAC) Model

2.7.1 Animals

Swiss albino mice (weight range 25–30g) were divided into normal, disease, positive and test groups, each consisting of six animals. The mice were kept in a room with air conditioning, with a cycle of 12 hours of light and darkness at 22±1°C and 55±1% humidity. They were constantly given access to water and fed a typical commercial rat pellet diet. Before the experiment began, the animals had a week to become acquainted with their new surroundings[18].

2.7.2 Transplantation of Cancer Cells

EAC cells were acquired from the National Centre for Cell Sciences (NCCS) in Pune, India. Every ten days, 2×10^6 cells suspended in PBS were transplanted intraperitoneally into each Swiss albino mouse to maintain the EAC cells *in vivo*. On the 7^{th} or 8^{th} day of cancer cell growth, ascites fluid was extracted from EAC cell-bearing animals and each test animal was given 0.1ml of cancer cell solution containing 2×10^6 cells intraperitoneally (i.p)[19].

2.7.3 Experimental Procedure

The mice were allocated to specific groups (with six mice in each group) and had unrestricted access to food and water. Only the mice in group I were intraperitoneally administered normal saline (5ml/kg body weight) and served as the control, whereas the mice in group II were designated as the disease control. Mice, excluding those in group I, were inoculated intraperitoneally with EAC cells at 2×10^6 cells per animal. Twenty-four hours after EAC cell administration, mice in group III were treated with Doxorubicin at a dose of 0.3mg/kg body weight intraperitoneally as a positive control and this treatment continued for nine consecutive days for the test groups. Following the final dose, the mice were subjected to an 18-hour fast and six mice from each group were euthanised through cardiac puncture to assess haematological and biochemical markers. The remaining mice were monitored for any changes in lifespan post-treatment[20].

2.7.4 Tumour Volume and Packed Cell Volume (PCV) Assessment

The ascitic fluid was extracted from the peritoneal cavity of the euthanised mice for volume measurement using a graduated cylinder. The ascitic fluid was centrifuged at 1,000 rpm and 4°C for 5 minutes to determine the PCV[21].

2.7.5 Evaluation of Viable and Non-viable Cancer Cells

The ascitic fluid was diluted 100-fold with Phosphate Buffered Saline (PBS), and a sample was placed in a Neubauer counting chamber. The cells were stained with 0.4% trypan blue in saline. Cells that absorbed the dye were counted as non-viable, whereas unstained cells were considered viable. The cell count was calculated by the formula: Cell count = (total number of cells × dilution factor) / (area × depth of the chamber)[22].

2.7.6 Analysis of Survival Metrics

The impact on survival was gauged by calculating the percentage increase in lifespan (% ILS) and the mean survival time (MST) using the formula: % ILS = [(MST of treated group / MST of the control group) - 1] × 100. The MST was determined by averaging the first and last mortality days in each group[23].

2.7.7 Haematological and Biochemical Analysis

Blood samples were analysed for haemoglobin levels and red and white blood cell counts, with differential white cell counts performed on Leishman-stained smears. For biochemical assessments, the blood was allowed to clot and the serum was separated by centrifugation at 2,500 rpm for 15 minutes. The serum levels of Glutamate Pyruvic Transaminase (SGPT), Glutamic Oxaloacetate Transaminase (SGOT), Alkaline Phosphatase (ALP), total proteins, and bilirubin were measured using established methods[24].

2.8 Histopathological Analysis

For histopathological analysis, liver samples from each experimental group were fixed in a 10% buffered formalin solution (pH 7.4) to ensure preservation. The preserved tissues were embedded in paraffin in the laboratory and thin sections of 5–10µm were prepared. The sections were then stained with hematoxylin and eosin for microscopic examination. The extent of hepatocyte necrosis was quantitatively assessed by analysing 10 randomly selected high-power fields per sample, using a grading scale from 0 to 5, where 0 indicates no necrosis, 1 signifies minimal necrosis in a few hepatocytes, 2 reflects necrosis in 10-24% of hepatocytes, 3 denotes necrosis in 25-39% of hepatocytes, 4 corresponds to necrosis in 40-49% of hepatocytes and 5 represents extensive necrosis in over 50% of hepatocytes[25].

2.9 Statistical Analysis

Graph pad prism software version 9.0 was used to calculate the significance level using appropriate statistical tools as needed.

3. Results

3.1 Preliminary Phytochemical Screening

The extract of *D. chloroxylon* was subjected to a preliminary phytochemical analysis, revealing several secondary metabolites, including alkaloids, saponins, flavonoids and tannins (Table 1).

3.2 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide (MTT) Assay

The findings indicated that the selected cancer cell lines were significantly cytotoxic after exposure to the *D. chloroxylon* Roxb. extract for 72 hours at 37°C and 5% CO_2 (Table 2). The reduction of the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan by mitochondrial enzymes in living cells was measured using the MTT colourimetric assay. The IC_{50} value determines the substance needed to disrupt 50% of cellular growth or survival of four cancer cell lines and one normal cell line. Breast cancer cell lines (MCF-7), lung cancer cell lines (A549), prostate cancer cell lines (DU 145), colon cancer cell lines (HT29), EAC and HUVEC were selected.

Table 1. Phytochemical screening of leaf extracts of *D. chloroxylon*

Phytochemicals	n-Hexane Extract	Ethanolic Extract
Alkaloids	+	+
Glycosides	-	-
Saponins	-	+
Flavonoids	-	+
Steroids	+	-
Tannins	-	+
Proteins	-	-
Carbohydrates	-	+
Amino acids	-	+

Present (+)/ Absent (-)

According to this analysis, the extract was cytotoxic to varying degrees in the four cancer cell lines. The IC_{50} value for EAC cells was determined to be 66.33±2.67µM. This comparatively lower IC_{50} value shows that the extract has strong cytotoxic effects on EAC cells. The IC50 value for HT29 cells was 37.23±2.16µM, which is lower than that for EAC cells, suggesting that the extract impacts the ability of these cells to grow or remain viable. The A549 cells also showed sensitivity towards the extract treatment with IC_{50} values of 73.27±2.11 µM. The extract exerted cytotoxic effects on MCF-7 cells, as evidenced by the fact that the IC_{50} value for these breast cancer cells was still relatively low (78.19±2.08µM).

The IC_{50} value for DU 145 cells was discovered to be 133.14±2.79µM, which is significantly greater than that for the other cancer cell lines, indicating that the extract is not sensitive to these prostate cancer cell lines. However, the IC_{50} value of the extract against HUVECs was 141.1±2.32µM,

showing that it is less hazardous to normal human cells. The IC_{50} values of Doxorubicin were substantially lower than those of the extracts. According to the findings, *D. chloroxylon* Roxb. is more potent against the two cancer cell lines (EAC and A549), with various degrees of cytotoxicity (Table 2).

3.3 Evaluation of Acute Oral Toxicity

The study on acute oral toxicity was conducted using Swiss Albino mice following guideline 423 provided by OECD. A limit dose of 2000mg/kg body weight was administered. Before the experiments, all the mice were fasted overnight with unlimited access to water. The mice were organised into five groups, each consisting of six mice. The first group acted as the control, receiving no test substance, whereas the second to fifth groups were administered varying doses of the test extracts orally at concentrations of 5, 50, 300, and 2000mg/kg body weight, respectively. The body weight of each mouse was recorded before administration of the doses to ensure an accurate dosage based on the individual body weights. After administration, the mice were closely monitored for any immediate toxicological effects within the first four hours and observed for three additional days. During this observation period, various parameters were assessed, including changes in body weight, urination patterns, food and water consumption, respiratory function, presence of convulsions or tremors, body temperature, gastrointestinal motility and changes in the appearance of the eyes and skin.

The extract showed no toxicity symptoms at 2,000mg/kg body weight. The $1/5^{th}$ of this dose i.e. 400mg/kg body weight, is selected as the therapeutic dose for future *in vivo* studies. (Table 3).

Table 2. IC_{50} values of *D. chloroxylon* extract and doxorubicin in MTT assay

Treatment	*D. chloroxylon*	Doxorubicin
MCF-7	78.19±2.08	1.21±1.19
A549	73.27±2.11	0.97±1.03
DU 145	133.14±2.79	2.03±1.75
HT29	37.23±2.16	2.03±1.05
EAC	66.33± 2.67	1.03±1.01
HUVECs	141.1±2.32	38.42±1.16

3.4 Effect on Tumour Parameters

3.4.1 Body Weight

The effect of *D. chloroxylon* on body weight was significant. The normal control group maintained the highest average body weight (36.24±1.38), indicating overall health. In contrast, the disease control group exhibited a significant decrease in body weight (31.78±1.46), reflecting the adverse effects of EAC. Both *D. chloroxylon* treated groups (200mg/kg and 400mg/kg) showed increased body weights (33.4±1.27 and 34.43±1.31, respectively) compared to the disease control group, suggesting that *D. chloroxylon* helped mitigate the weight loss associated with EAC (Table 4 and Figure 1).

3.4.2 Tumour Volume

Tumour volume analysis revealed the antitumor efficacy of the *D. chloroxylon*. The disease control group had a substantial tumour volume (5.41±0.21 ml). However, the *D. chloroxylon* treatments at both dosages significantly reduced tumour volume, with the 400mg/kg dosage (2.12±0.06 ml) being more effective than the 200 mg/kg dosage (3.52±0.42 ml). This reduction indicates a dose-dependent effect of *D. chloroxylon* Roxb. in controlling tumour growth. (Table 4 and Figure 1).

3.4.3 Packed Cell Volume

Packed cell volume in the *D. chloroxylon*-treated groups showed encouraging results. While the disease control group displayed a higher volume (3.62±0.14 ml), indicative of more cancer cells, both dosages of *D. chloroxylon* (200mg/kg and 400mg/kg) considerably reduced this volume (1.58±0.19 ml and 0.71±0.08 ml, respectively). These findings suggest that *D. chloroxylon*

Table 3. Effect of *D. chloroxylon* on the body weight of mice

Treatment	*D. chloroxylon* Ethanolic Extract		
	Day 0	Day 7	Day 14
Control	33.6±0.21	36.5±0.28	38.4±0.24
5mg/kg	31.7±0.11	34.6±0.19	37.4±0.22
50mg/kg	35.5±0.25	37.4±0.38	39.4±0.35
300mg/kg	31.7±0.24	33.6±0.16	35.5±0.17
2000mg/kg	35.5±0.34	37.4±0.54	40.3±1.06

treatment effectively reduced the severity of cancer in mice. (Table 4 and Figure 1).

3.4.4 Viable Cell Count

The viable cell count results highlighted the anticancer properties of *D. chloroxylon*. The disease control group had a high count of viable cancer cells (9.77±0.75 million cells/mL), whereas the *D. chloroxylon* treated groups showed a marked reduction in these cells. Notably, the 400mg/kg *D. chloroxylon* dosage (1.41±0.12 million cells/mL) was more effective than the 200mg/kg dose, indicating *D. chloroxylon* potential in reducing the number of viable cancer cells in a dose-dependent manner. (Table 4 and Figure 1).

3.4.5 Non-viable Cell Count

Analysis of non-viable cell counts further emphasised the effectiveness of *D. chloroxylon*. While the disease control group had a lower count of dead cancer cells (0.37±0.11 million cells/mL), the *D. chloroxylon* Roxb. treated groups exhibited higher counts (0.67±0.16 million cells/mL for 200mg/kg and 0.77±0.1 million cells/mL for 400mg/

Table 4. Effect on tumour parameters

Treatment	Body Weight	Tumor Volume (ml)	Packed Cell Volume (ml)	Viable Cell Count (cells×10^6 /mL)	Non-Viable Cell Count (cells×10^6 / mL)	Mean Survival Time (days)	Percentage increase in lifespan (ILS%)
Normal Control	36.24±1.38	0	0	0	0	0	0
Disease Control	31.78±1.46	5.41±0.21	3.62±0.14	9.77±0.75	0.37±0.11	18.5±1.76	0
Doxorubicin	35.15±1.34***	0.19±0.09****	0****	0.35±0.04****	1.43±0.25****	39.5±1.38****	91
D. chloroxylon (200mg/kg bw)	33.4±1.27 ^{ns}	3.52±0.42****	1.58±0.19****	4.18±0.48****	0.67±0.16**	25±0.63****	35.1
D. chloroxylon (400mg/kg bw)	34.43±1.31***	2.12±0.06****	0.71±0.08****	1.41±0.12****	0.77±0.1***	37.5±0.84****	70.3

All values are expressed as Mean ± SEM, statistical analysis by one-way ANOVA followed by Dunnett's test, compared with the disease group. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05, ^{ns}Non-significant

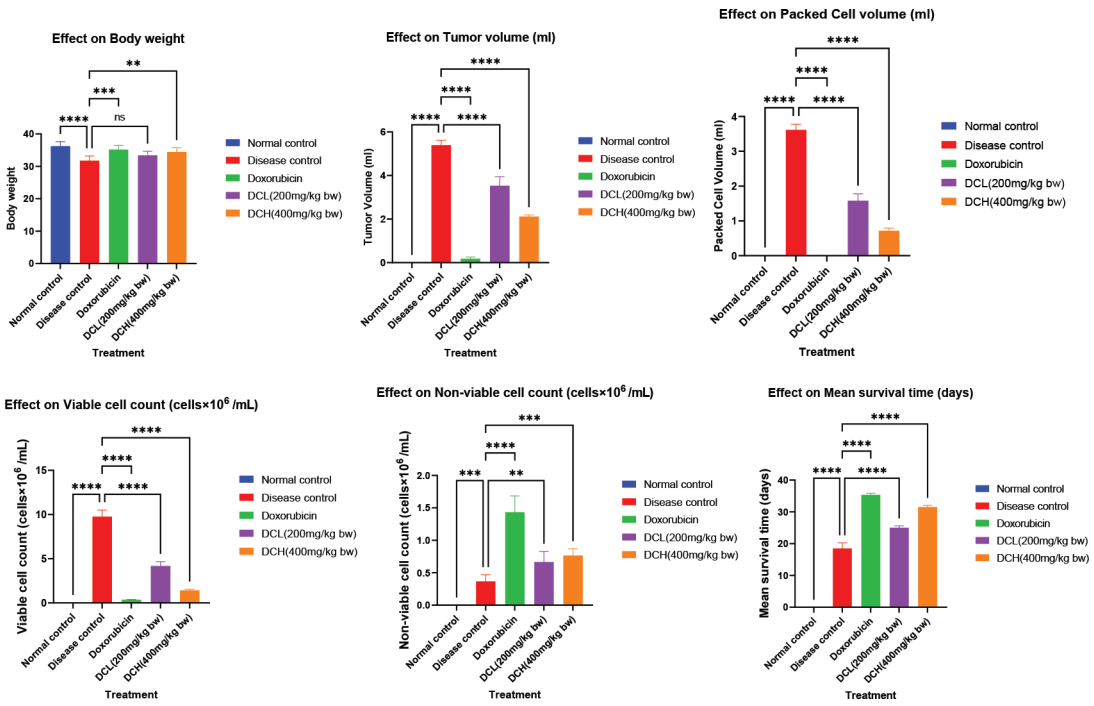

Figure 1. Effect of *D. chloroxylon* on tumour parameters.

kg). This increase in the non-viable cell count with *D. chloroxylon* Roxb. treatment suggests that it effectively induces cancer cell death. (Table 4 and Figure 1).

3.4.6 Mean Survival Time

The mean survival time of mice was significantly influenced by *D. chloroxylon* treatment. The disease control group had the lowest survival time (18.5±1.76 days), reflecting the lethal nature of EAC. Conversely, *D. chloroxylon* treatment improved survival times (25±0.63 days for 200mg/kg and 37.5±0.84 days for 400 mg/kg), significantly increasing the 400mg/kg dose, indicating that *D. chloroxylon* has a substantial impact on prolonging the lifespan of EAC-bearing mice. (Table 4 and Figure 1).

3.4.7 Percentage Increase of Lifespan (ILS%)

In terms of the percentage increase in lifespan, *D. chloroxylon* showed promising results. The disease control group showed no increase in lifespan (0%). However, *D. chloroxylon* treatment led to a significant increase, particularly at the 400mg/kg dosage (70.3%) and, to a lesser extent, at the 200mg/kg dosage (35.1%), demonstrated the potential of *D. chloroxylon* in extending the life expectancy of mice affected by EAC. (Table 4 and Figure 1).

3.5 Effect on Haematological Parameters

Diospyros chloroxylon extract positively influenced various haematological parameters. It improved haemoglobin content, red blood cell and white blood cell counts and normalised the levels of monocytes, lymphocytes and neutrophils (Table 5). These effects are crucial since cancer often leads to haematological imbalances (Figures 2 and 3).

3.5.1 Hb Content (Haemoglobin Content)

The Hb content is a key indicator of the oxygen-carrying capacity of blood. The normal control group in the study exhibited a healthy Hb level (13.83±0.96g/dL), indicating good overall blood health. In contrast, the disease control group showed a significant reduction in Hb content (6.63±0.37g/dL), likely due to the impact of EAC, such as cancer-associated anaemia. The *D. chloroxylon*-treated groups displayed an improvement in Hb levels compared to the disease control, with the 400mg/kg dosage (9.61±0.41g/dL) more effective than the 200mg/kg dosage (8.04±0.41g/dL) suggesting that *D. chloroxylon* contributes positively to maintaining or improving haemoglobin levels in the presence of EAC.

3.5.2 RBC (Red Blood Cell Count)

Red blood cells are crucial for oxygen transport. The normal control group maintained a healthy RBC count (5.00±0.27 million cells/µL), while the disease control group exhibited a lowered RBC count (2.91±0.17 million cells/µL), reflecting EAC's negative impact, possibly due to reduced RBC production or increased destruction. The *D. chloroxylon* treatments increased RBC counts, especially at the 400mg/kg dosage (3.53±0.09 million cells/µL), suggesting that *D. chloroxylon* helps restore or maintain RBCs in EAC-affected mice.

3.5.3 WBC (White Blood Cell Count)

White blood cells are vital for immune response. The normal control group showed a normal WBC count (4.55±0.24 thousand cells/µL). The disease control group had an elevated count (8.99±0.48 thousand cells/µL), possibly due to the immune system's response to EAC. Interestingly, *D. chloroxylon* treatment, especially the 400mg/kg dosage (5.60±0.17 thousand cells/µL),

Table 5. Effect on haematological parameters

Treatment	Hb Content	RBC	WBC	Monocytes	Lymphocytes	Neutrophils
Normal Control	13.83±0.96	5.00±0.27	4.55±0.24	1.97±0.27	74.41±3.5	21.97±1.04
Disease Control	6.63±0.37	2.91±0.17	8.99±0.48	1.41±0.2	33.32±1.9	87.73±5
Doxorubicin	12.30±0.83****	3.84±0.45****	4.97±0.12****	1.77±0.25*	69.68±3.78****	28.91±3.41****
D.chloroxylon (200mg/kg bw)	8.04±0.41**	2.99±0.07^{ns}	6.59±0.2****	1.52±0.21^{ns}	40.80±1.57***	71.40±3.59****
D.chloroxylon (400mg/kg bw)	9.61±0.41****	3.53±0.09**	5.60±0.17****	1.64±0.23^{ns}	56.98±3.26****	38.18±1.19****

All values are expressed as Mean ± SEM, statistical analysis by one-way ANOVA followed by Dunnett's test, compared with the disease group.
****P<0.0001, ***P<0.001, **P<0.01, *P<0.05, ^{ns}Non-significant

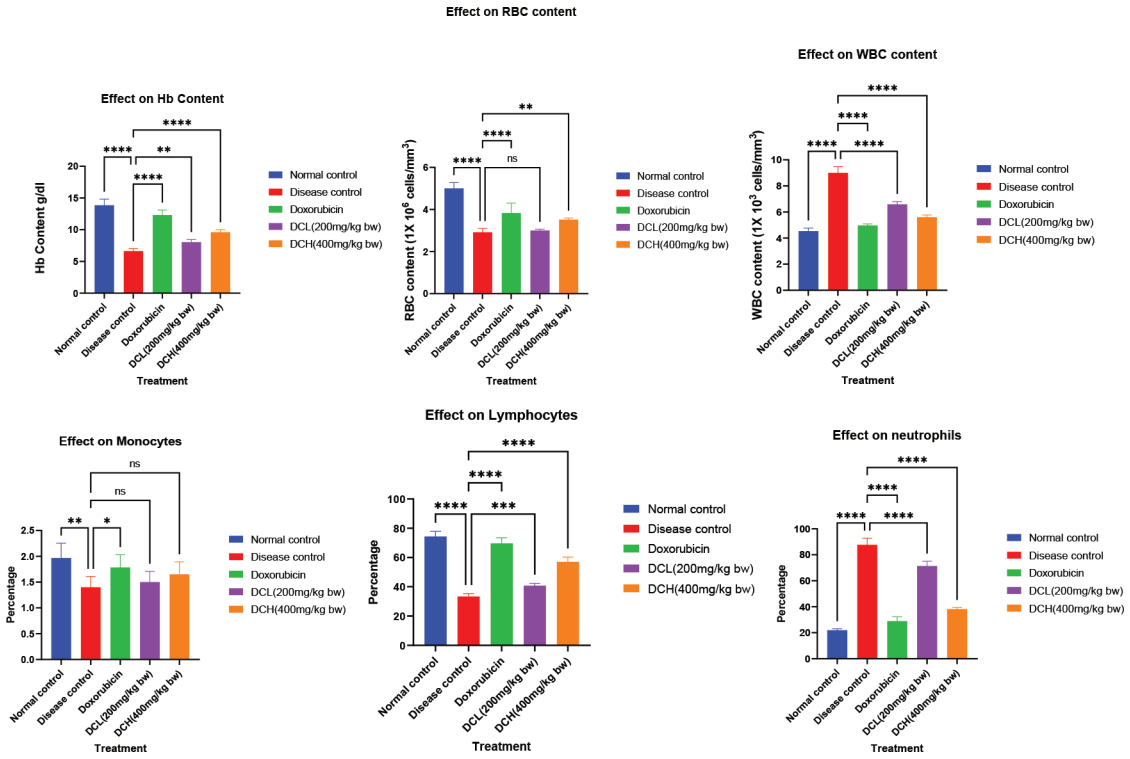

Figure 2. Effect of *D. chloroxylon* Roxb. on haematological parameters.

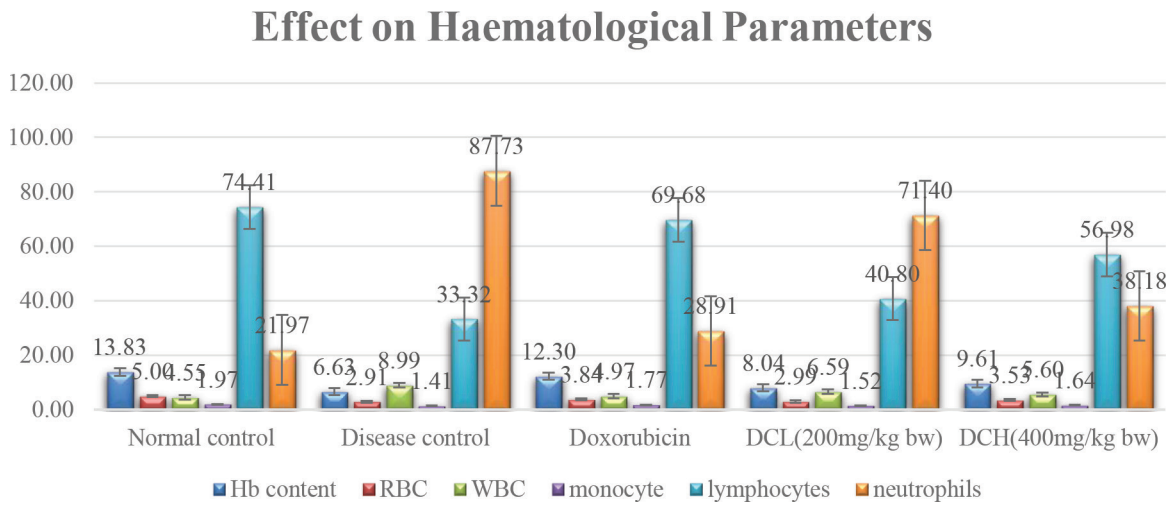

Figure 3. Effect of *D. chloroxylon* Roxb. on haematological parameters.

helped reduce the WBC count towards normal levels, indicating its potential in modulating the immune system in the context of EAC.

3.5.4 Monocyte Count

Monocytes are a type of white blood cell involved in immune response. The normal control group had a standard monocyte count (1.97±0.27%), whereas the disease control group experienced a slight decrease (1.41±0.2%), possibly due to EAC's impact on the immune system. *D. chloroxylon* treatment slightly increased the monocyte count in both dosages (1.52±0.21% for 200mg/kg and 1.64±0.23% for 400mg/kg), indicating a potential stabilising effect of *D. chloroxylon* on monocyte levels in EAC conditions.

3.5.5 Lymphocyte Count

Lymphocytes play a critical role in the immune system. The normal control group showed a high percentage of lymphocytes (74.41±3.5%), typical of a healthy immune system. The disease control group had a significantly reduced percentage (33.32±1.9%), reflecting the immunosuppressive effects of EAC. *D. chloroxylon* treatment improved lymphocyte percentages, particularly with the 400mg/kg dosage (56.98±3.26%), suggesting that *D. chloroxylon* might bolster immune function or counteract EAC's immunosuppressive effects.

3.5.6 Neutrophil Count

Neutrophils are key components of the innate immune system. In the normal control group, neutrophil count was within the normal range (21.97±1.04%). The disease control group showed a substantial increase (87.73±5%), likely due to the body's inflammatory response to EAC. Notably, *D. chloroxylon* treatment, especially at 400mg/kg (38.18±1.19%), significantly reduced neutrophil count, indicating its potential to modulate the inflammatory response typically seen in EAC.

3.6 Effect on Biochemical Parameters

3.6.1 SGOT (Serum Glutamic- Oxaloacetic Transaminase) and SGPT (Serum Glutamic-Pyruvic Transaminase)

SGOT and SGPT are enzymes indicative of liver health. In this study, the disease control group showed significantly elevated levels of SGOT (78.18±3.68 IU/L) and SGPT (74.12±1.36 IU/L), which is typical in cancer due to liver stress or damage. The *D. chloroxylon* treatment, at both 200mg/kg and 400mg/kg dosages, resulted in lower levels of SGOT (52.87±1.89 IU/L and 48.64±1.74 IU/L, respectively) and SGPT (62.36±1.82 IU/L and 48.38±2.41 IU/L, respectively) compared to the disease control. This reduction, though not to the normal levels, indicates a hepatoprotective effect of *D. chloroxylon* suggesting that it may mitigate liver damage associated with EAC.

3.6.2 SALP (Serum Alkaline Phosphatase)

Elevated SALP levels, as seen in the disease control group (116.04±2.18 IU/L), can indicate liver dysfunction or changes in bone metabolism. The *D. chloroxylon* treatments at both dosages reduced SALP levels (93.24±2.45 IU/L for 200mg/kg and 85.78±2.26 IU/L for 400mg/kg) compared to the disease control. It suggests that *D. chloroxylon* may have a protective effect on the liver and bones, helping to maintain their function in the presence of EAC.

3.6.3 Total Protein

Total protein levels are important for assessing nutritional status and liver function. The disease control group showed a significant decrease in total protein levels (4.49±0.24 mg/dL), a common issue in cancer due to malnutrition or impaired liver function. The *D. chloroxylon* treatment improved total protein levels (6.28±0.15mg/dL for 200mg/kg and 7.19±0.15 mg/dL for 400mg/kg), though still below normal levels, indicating *D. chloroxylon* Roxb. may help improve protein synthesis or reduce protein loss associated with EAC, contributing to better overall health in treated mice.

3.6.4 Bilirubin

Bilirubin levels are a marker for liver function, with elevated levels indicating liver stress or damage. The disease control group exhibited high bilirubin levels (3.60±0.21mg/dL). The *D. chloroxylon* treatments at both dosages showed a reduction in bilirubin levels (2.88±0.16mg/dL for 200mg/kg and 2.38±0.14mg/dL for 400mg/kg) compared to the disease control. While these levels are higher than those in the normal control, the reduction indicates that *D. chloroxylon* may positively affect liver health in EAC, possibly by reducing liver damage or improving bilirubin metabolism (Table 6, Figures 4 and 5).

3.7 Histopathology

The histopathology report reveals varied responses across different groups in the EAC model. The normal control group displayed a healthy liver with a well-organised tissue structure and no inflammation or necrosis, serving as the baseline for comparison. In contrast, the disease control group, representing the EAC model, exhibited enlarged hepatic sinusoids and disrupted cellular arrangements, indicative of altered blood flow and liver damage caused by the carcinoma. Cell death and a loss of the typical hepatocyte

Table 6. Effect on biochemical parameters

Treatment	SGOT(IU/L)	SGPT (IU/L)	SALP (IU/L)	Total Protein (mg/dL)	Bilirubin (mg/dL)
Normal Control	32.08±1.52	27.72±0.79	76.06±1.27	8.82±0.47	1.13±0.1
Disease Control	78.18±3.68	74.12±1.36	116.04±2.18	4.49±0.24	3.60±0.21
Doxorubicin	35.91±1.78****	31.67±0.9****	76.98±1.7****	7.43±0.39****	1.55±0.13****
D. chloroxylon 200mg/kg bw)	52.87±1.89****	62.36±1.82****	93.24±2.45****	6.28±0.15****	2.88±0.16****
D. chloroxylon (400mg/kg bw)	48.64±1.74****	48.38±2.41****	85.78±2.26****	7.19±0.15****	2.38±0.14****

All values are expressed as Mean ± SEM, statistical analysis by one-way ANOVA followed by Dunnett's test, compared with the disease group. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05, ^{ns}Non-significant

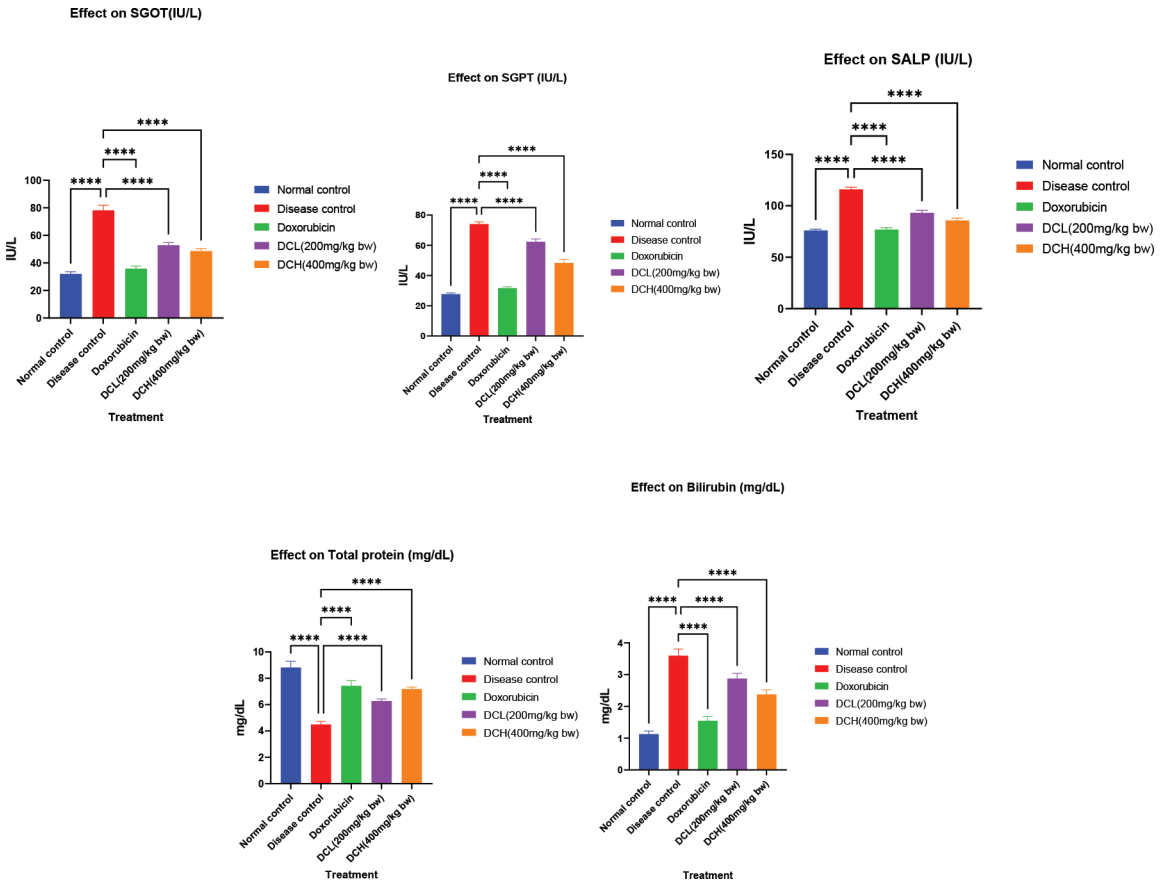

Figure 4. Effect of *D. chloroxylon* on biochemical parameters.

cord structure further pointed to the malignancy's detrimental effects on liver function.

The positive control group, treated with Doxorubicin, showed significant structural improvements in the hepatic tissue, yet some issues like dilated sinusoids and occasional necrotic cells persisted, suggesting partial therapeutic effectiveness. Treatment with *D. chloroxylon* at a low dose (200mg/kg bw) had limited effect in addressing tumour-induced liver damage, with ongoing issues like dilated sinusoids and cytomegaly.

However, a higher dose of *D. chloroxylon* (400mg/kg bw) markedly improved the liver's structure and function, evidenced by the re-establishment of organised hepatic cords and reduced necrosis. Still, minor sinusoidal dilation was observed indicating a significant, albeit not complete, reversal of carcinoma-induced liver damage (Figure 6).

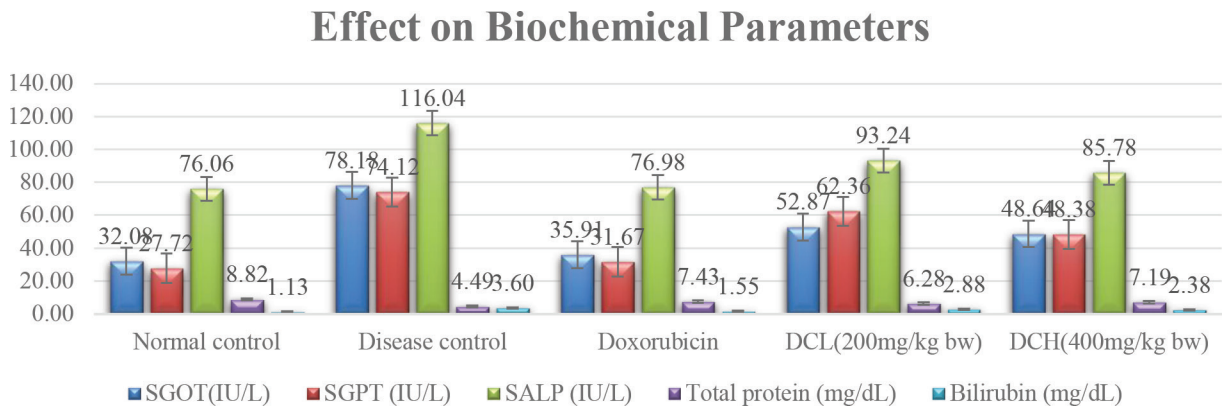

Figure 5. Effect of *D. chloroxylon* on biochemical parameters.

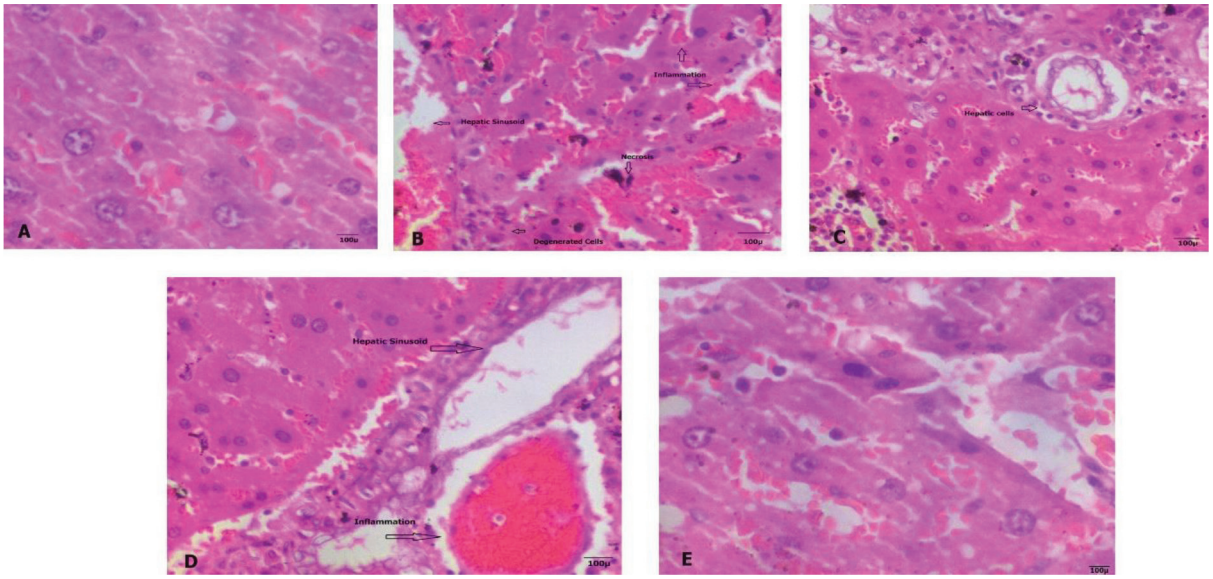

Figure 6. Histopathology of the liver.

4. Discussion

The MTT assay revealed significant cytotoxic effects of *D. chloroxylon* extract on various cancer cell lines, including EAC, by reducing the tetrazolium dye MTT to formazan, indicating mitochondrial activity in living cells. The IC_{50} values, which measure the concentration needed to inhibit 50% of cellular growth, varied among different cancer cell lines, with a notably lower value for EAC cells, suggesting a strong cytotoxic effect of the extract on EAC cells. While the extract was less effective on other cancer cell lines like A549 (lung), MCF-7 (breast), DU 145 (prostate) and HT29 (colon) it showed comparatively lesser toxicity towards HUVECs, indicating a degree of selectivity in its cytotoxic action.

The study on *D. chloroxylon* leaf extract in EAC mice models showed significant tumour-related impacts. The extract effectively mitigated weight loss associated with EAC, indicating its potential to counteract cancer-associated cachexia. Furthermore, *D. chloroxylon* demonstrated a dose-dependent reduction in tumour volume, suggesting active compounds that inhibit tumour growth. It is complemented by decreased packed cell volume and changes in viable and non-viable cell counts in the *D. chloroxylon* treated groups, highlighting the extract's potential to reduce tumour severity and induce cancer cell death.

The haematological findings from the study are equally significant. *D. chloroxylon* treatment improved haemoglobin levels and red blood cell count, indicating

its beneficial role in ameliorating cancer-induced anaemia and enhancing oxygen transport. Moreover, the normalisation of white blood cell counts, including monocytes, lymphocytes and neutrophils, with *D. chloroxylon* treatment reflects its immunomodulatory effects. This normalisation is crucial, considering the dysregulation of the immune system often observed in cancer.

Biochemically, *D. chloroxylon* leaf extract showed a hepatoprotective effect in EAC-affected mice. Although not normal, the reduction in liver enzyme levels (SGOT and SGPT) and SALP suggests its role in mitigating liver stress or damage common in cancer. Additionally, improving total protein levels and reducing bilirubin levels with *D. chloroxylon* treatment underlines its supportive role in maintaining liver function and overall nutritional status, which are critical aspects of cancer management.

Histopathology shows diverse treatment effects. The normal control group had a healthy, organised liver, while the disease control group showed liver damage with enlarged sinusoids and disrupted cell arrangement due to the carcinoma. Doxorubicin (positive control) improved the liver structure but did not fully eliminate abnormalities like dilated sinusoids and necrotic cells. Low-dose *D. chloroxylon*. (200mg/kg) partially addressed liver damage, but some issues remained. A higher dose (400mg/kg) significantly enhanced liver structure and function, reducing necrosis but not completely resolving sinusoidal dilation, indicating a substantial yet incomplete recovery from carcinoma-induced damage.

5. Conclusion

In conclusion, *D. chloroxylon* leaf extract exhibits multiple therapeutic potentials in cancer treatment. The convergence of *in vitro* cytotoxicity data with *in vivo* therapeutic outcomes underlines the potential of *D. chloroxylon* as an effective anticancer agent. Its ability to selectively exert cytotoxic effects on cancer cells, particularly EAC cells, while showing reduced toxicity towards normal cells is noteworthy. Its ability to reduce tumour burden, improve haematological and biochemical parameters and potentially extend survival times positions it as a promising candidate for further research in cancer therapy. However, based on animal models, these promising findings necessitate further investigation, including human clinical trials, to fully establish its efficacy and safety for potential use in human cancer treatment.

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7. References

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