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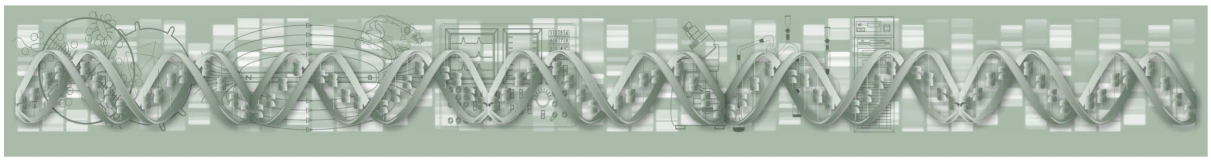

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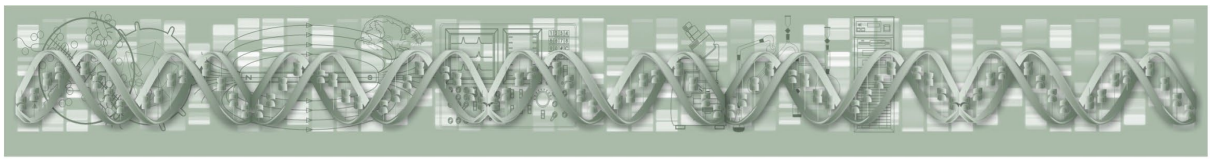

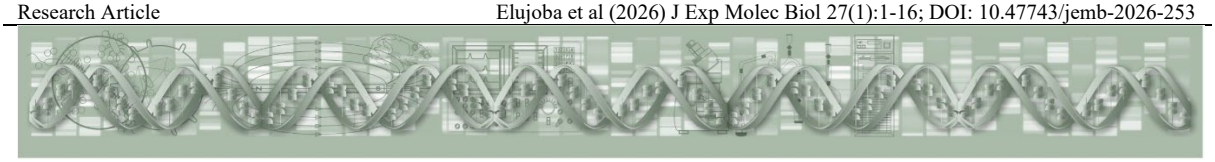

BCL-2 GENE EXPRESSION EFFECT ON HAEMATOLOGICAL PROFILES AMONG CML PATIENTS IN ILE –IFE. OSUN STATE. NIGERIA

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Abstract

B-cell lymphoma 2 (BCL-2) was the first gene identified to inhibit programmed cell death and function as an anti-apoptotic regulator. Its anti-apoptotic activity represents a major oncogenic mechanism in haematological malignancies, largely driven by the aberrant upregulation of BCL-2. This overexpression arises from multiple underlying mechanisms. BCL-2 gene is located on chromosome 18q21.33. It was first discovered by cloning the breakpoint region of the t(14;18) translocation, a chromosomal abnormality commonly found in follicular lymphomas (FL). Also established is BCL-2 MicroRNA (miRNA) deregulation, which leads to increased BCL-2 expression in the incidence of chronic lymphocytic leukemia (CLL) and CML is associated with increased Bcl-2 expression at the protein and mRNA levels. The intrinsic (mitochondrial) pathway of apoptosis is tightly regulated by the balance of pro-apoptotic and anti-apoptotic BCL-2 family proteins. Dysregulation of this system shift the balance toward cell survival, an important step in many hematologic malignancies. The mechanisms of BCL-2 alteration in Hematologic malignancies include genetic alterations, aberrant signaling pathways, infectious agents and environmental exposure etc. The aim of this study is to assess BCL-2 gene alterations through its expression levels and to evaluate their effects on haematological profiles among Chronic Myelogenous Leukemia (CML) patients in Ile-Ife. This investigation is particularly important given the scarcity of research on the role of the BCL-2 gene in the development of CML, especially among Nigerian cancer patients. To evaluate the relationship between BCL-2 expression levels and haematological parameters for their potential diagnostic relevance in CML patients. To compare the expression of the BCL-2 gene and haematological parameters in patients with CML and apparently healthy individuals and to a**ssess** the potential of BCL-2 gene expression as a molecular marker and possible driver

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in the pathogenesis of haematological malignancies, particularly CML. This study was cross-sectional and a total of 100 consenting participants were recruited: 50 known CML patients and 50 control subjects. Among the CML participants, the largest proportion (42%) were between 41 and 60 years old, while only a small fraction were within the 81–90-year age range. Females accounted for 58% of this group. Most participants (86%) had attained tertiary education, whereas 6% had no formal education. In the control group, the predominant age category was 21–40 years, and males constituted 60% of the participants. Nearly half of the controls (48%) had secondary-level education. All CML participants were on CML-specific treatment. Among them, 16% commenced therapy in 2008, 14% in 2013, 18% in 2017, and 26% began treatment in 2021, with all continuing their therapy up to the study period in 2024. Full blood count was done using 3-part haematology auto analyzer (Mindray), while RNA extraction and qPCR Bcl-2 quantification were done using real time PCR equipment. In this study, participants' age, sex, level of education, and CML-specific treatment did not have a significant impact on the study outcomes. The results demonstrated that BCL-2 gene expression was significantly higher in CML samples compared to controls ($p < 0.05$), suggesting that dysregulated BCL-2 may play a role in the pathogenesis of CML. Haematological parameters were also significantly affected by BCL-2 expression ($p < 0.05$). Specifically, 30% of CML patients with BCL-2 overexpression exhibited leukocytosis, compared to normal WBC counts in 43 (86%) of the controls and 29 (58%) of CML patients with no gene expression. Additionally, more than half (24%) of CML patients with BCL-2 overexpression had anaemia, whereas the majority of CML patients without expression (44%) and controls (70%) had normal PCV values. Thrombocytosis of 14% was observed in CML patients with gene expression, which was compared to 2% in those without gene expression and 4% in the control group, indicating a prognostic significance. The overexpression of BCL-2 observed in this study is characteristic of CML and its association with anaemia, leukocytosis and neutrophilia proves that Bcl-2 expression level is a marker for the role of BCL-2 gene in the incidence of CML disease and can be used in the stratification and evaluation of hematologic malignant (CML) disease. Targeting a driver of haematologic malignancies (H.M.) is an effective approach to identify possible prognosis for these diseases and aid in the BCL-2 therapy.

Keywords*:* B-Cell Lymphoma 2 gene, chronic myelogenous leukemic, leucocytosis, anaemia, neutrophilia

Introduction

B Cell Lymphoma-2 (BCL-2) is one of the Bcl-2 protein family encoded by the BCL-2 gene[1]. It was the first anti-death gene, anti-apoptotic modulator associated with cancer.[2] BCL-2 was also the first gene demonstrated to prolong cell survival without promoting increased proliferation[3-4]. The suppression of apoptosis mediated by BCL-2 represents a critical step in tumorigenesis[3-4]. BCL-2gene is located on chromosome 18q21.33. It was first discovered by cloning the breakpoint region of the t (14;18) translocation, a chromosomal abnormality commonly found in follicular lymphomas (FL).[5] Bcl-2 resides within the mitochondria, the cell's powerhouse. The BCL-2 protein is the founding member of the BCL-2 family of apoptosis regulators. It functions by neutralizing pro-apoptotic proteins like Bax and Bak, preventing them from triggering the release of cytochrome c, a crucial step in the intrinsic apoptotic pathway. Mimetics (BH3-only proteins) circumvent BCL-2 and BCL-XL, sequester and inhibit it, however, freeing BAX and BAK to initiate the cascade caspase leading to cell death.[6] this delicate balance between pro- and anti-apoptotic proteins maintains cellular homeostasis. However, when Bcl-2 expression surpasses a certain threshold, it disrupts this balance, enabling cell survival and hindering apoptosis[7-8]. Mechanisms underlying BCL-2 dysregulation in hematologic malignancies include gene mutations, aberrant signaling pathways, infectious

agents, and environmental exposures. For example, elevated BCL-2 expression is observed in Multiple Myeloma (MM), particularly in patients carrying the t(11;14) translocation[9]. In Chronic Lymphocytic Leukemia (CLL), the loss or downregulation of miR-15a and miR-16-1, which normally suppress BCL-2 mRNA, results in increased BCL-2 expression[10-11]. Studies also indicate that BCL-2 overexpression is more common in Acute Myeloblastic Leukemia (AML) patients with specific cytogenetic abnormalities, such as t(8;21)[12-13]. Additionally, certain oncogenic viruses can directly or indirectly induce BCL-2 upregulation in hematologic contexts[14-15]. Benzene exposure has been shown to induce BCL-2 upregulation contributing to hematotoxicity and luekemogenic transformation[16]. Bcl-2 is highly expressed in erythroid precursors which promotes their survival, which might disrupt their apoptosis, influencing iron recycling, impacting overall iron homeostasis and eventual haemoglobin synthesis disruption[17],which leads to an increase in the production of macrocytosis and slight increase in mean cell volume (MCV) and possibly mean cell haemoglobin (MCH)[18] Bcl-2 overexpression has been associated with the development of lymphoid malignancies, such as chronic lymphocytic leukemia (CLL)[19] as it promotes B-lymphocyte survival, contributing to an abnormal increase in circulating lymphocytes, a hallmark of the disease. CML is associated with increased BCL-2 expression at the protein and mRNA levels, leading to uncontrolled proliferation of leukemic cells[20]. The BCR-ABL1 oncogene activates various signaling pathways, including NF-κB and PI3K/Akt, which upregulate Bcl-2 transcription and protein stability [21]. This elevated Bcl-2 confers resistance to apoptosis in CML blasts, promoting their survival and disease progression (Wang et al. 2017)[22]. Studies suggest that Bcl-2 dysregulation could promote megakaryocyte survival and differentiation, potentially leading to increased platelet production[23]. So this finding has established the direct proportion between leukocytosis, neutrophilia, lymphopenia, anaemia and BCL-2 overexpression in the diagnosis of CML. The aim of this study is to assess BCL-2 gene alterations through its expression levels and to evaluate their effects on haematological profiles among Chronic Myelogenous Leukemia (CML) patients in Ile-Ife. The objectives are to evaluate the relationship between BCL-2 expression levels and haematological parameters for their potential diagnostic relevance in CML patients, to compare the expression of the BCL-2 gene and haematological parameters in patients with CML and apparently healthy individuals and to assess the potential of BCL-2 gene expression as a molecular marker and possible driver in the pathogenesis of haematological malignancies, particularly CML. This investigation is particularly important given the scarcity of research on the role of the BCL-2 gene in the development of CML, especially among Nigerian cancer patients.

Materials and Methods

Study Area

The study was carried out in Obafemi Awolowo University Teaching Hospitals Complex (OAUTHC), Ile-Ife, and Seventh Day Adventist Hospital (SDAH), Ile-Ife, Osun State, South West, Nigeria. Ile-Ife is about 218 kms northeast of Lagos, 40km from Osogbo. Ife is Latitude 7°28′N and 7°45′N and longitudes 4°30′E and 4°34′E, east of the city of Ibadan. It has a population of 501,000 people[24]. OAUTHC is along Ilesa road while SDA is at Idiomo in between Mayfair and Lagere, Ile-Ife (Ife central local government).

Study Design

The study is a cross-sectional study representing a random sampling of the haematological malignant patients in Ife metropolis. The research was designed to assess the BCL2 expression effect on haematologic profiles among CML patients in Ile-Ife. This study was carried out within three months.

Study Population and Subjects

The participants were known Chronic myelogenous leukemic (CML) patients who attend the referral haematology clinic. 50 CML patients were recruited for the study. Among the CML patients, 42% were aged between 41 and 60 years, with 6% participants in the 6 – 20 age range and 2% participants in the 81–90 age range. Females accounted for more than half of the CML group (58%). Most participants 86% had attained tertiary education, while only 6% had no formal education. The control group were prospective healthy subjects with no history of haematologic malignancy. 50 control subjects were recruited. They were screened before being recruited. Among the control group, the predominant age range was 21–40 years, with males representing the larger proportion 60%, and approximately half 48% have completed secondary education.

Inclusion and Exclusion criteria

Inclusion: Known/diagnosed CML patients who attended haematology clinic were allowed for the study

Newly diagnosed CML patients

All CML patients on treatment with CML drugs

CML patients irrespective of age were recruited

Exclusion: Other haematological malignancy excluded

CML patients on Mimetics drug or any other drug that repress BCL-2

No other associated ailment was considered

Sample size determination

Using formula validated by Adebola et al.[25] the sample size was determined as follows:

$n = Z \times 2 \times p \times q/d^2$

(P = prevalence, Q=1-P, d – degree of accuracy (0.05), Z = (1.96), P=0.22, d=0.05, confidence level: 95%).

According to Otu and Ejike, (2021), the prevalence of Haematologic Malignancies in Abuja was 6.66%

P= prevalence of Haematologic malignancies - 6.66%[26]

Then the formular will be considered: Z= 1.96 (for 95% confidence level) d= 0.05 (confidence interval or tolerance error)

N= Sample size $N = \frac{Z \times 2 \times P(1-P)}{d^2}$

$= \frac{1.96 \times 2 \times 0.067(1-0.067)}{0.05^2}$

$= \frac{3.92 \times 0.067 \times 0.933}{0.0025} = 98.02$

Therefore, the minimum sample size-N is pegged at 100.

Ethical considerations

Ethical clearance was obtained from the review board of Seventh Day Adventist Hospital (SDAH), Ile-Ife, Osun state (SERC-2024-3-0035). All research procedures were conducted in accordance with the ethical standard of the responsible committees of the two hospitals and in line with the principles outlined in the WMA Declaration of Helsinki.

Informed Consent**:** All participants were provided with detailed written information outlining the purpose of the study, the procedures involved and anticipated benefits.

Questionnaire was administered to obtain written informed consent from each participant before their enrollment. Participants were informed of their right to withdraw from the study at any point without penalty.

Confidentiality: To ensure confidentiality, all participant data were anonymized using unique identifiers. Only authorized members of the research team had access to the data. Electronic

data were stored on password-protected computer laptop, while physical documents were securely stored in locked filing cabinets.

Participant Well-being: Participants retained the right to withdraw from the study at any time without consequence. Any adverse events associated with blood sample collection, such as bruising or hematoma, were addressed promptly by qualified medical personnel in charge. Participants were also provided with the contact information of the principal investigator and the Institutional Review Board (IRB) to address any concerns or inquiries related to the study. The whole research is self-funded, no conflict of interest and no risk involved.

Blood samples collection, storage and transportation

About 4 mL venous blood samples were collected, 2 mL were dispensed into two anticoagulated (EDTA- Ethylene–diamine tetraacetic acid) sample bottles each, i.e., 2 sample bottles containing 2ml of blood each for each participant (test and control). One blood sample was meant for full blood count while the second was for RNA extraction and qPCR analysis. Samples were well mixed after collection and labelled appropriately. 200 blood samples were collected in total. 50 blood samples from CML patients and 50 blood samples from the control were used for full Blood Count, blood samples were analysed within 8 hours of collection using "auto analyzer machine (3 parts)". Another blood samples from CML patients and the control respectively meant for RNA extraction and qPCR analysis were kept at -4°C to prevent haemolysis until sample size 100(50 CML + 50 CONTROL) were realized. The blood sample size-100(50 CML + 50 CONTROL) were transported in cold chain (2 to 8°C) to Biorepository and Clinical Virology Laboratory, College of Medicine, University of Ibadan for RNA extraction and eventual RT-PCR analysis for BCL-2 gene expression.

Full Blood Count (FBC) Using (Mindray) Haematology Auto Analyzer

Procedure: Blood samples were placed on blood mixer machine. One after the other, the blood sample was put under the probe of the autoanalyzer. Auto analysis was done for the following parameters: PCV, Total and Absolute WBCs count, Platelet count, and Red Cell indices.

RNA Extraction Protocols (Qiagen Kit)

Arranged in a plastic rack were 2 mL Eppendorf tube according to the number of the expected samples to be processed. 560 µl of the prepared AVL buffer containing 5.6 µl of carrier RNA (probe) was dispensed into the arranged and labelled Eppendorf tube. Into each of the Eppendorf tube, 140 µl of the fresh or frozen blood samples were dispensed into corresponding labelled tube. Each sample was vortex for 10 secs and spun down briefly using the microcentrifuge and incubated at room temperature for 10 mins. 560 µl of absolute ethanol 96% -100% was added into each labelled tube containing lysed samples (lysis buffer + sample + carrier RNA) (inactivation). Each sample was vortexed for 10secs and spun down briefly using the microcentrifuge placed in the biosafety cabinet. 630 µl of each lysate (sample + lysis buffer + ethanol) was aspirated into corresponding labelled spin column, and centrifuged for 1 min at 12000 rpm. Collection tubes were discarded and each spin column was placed into a new collection tube. The remaining 630 µl of each lysate (sample + lysis buffer + ethanol) was aspirated into corresponding labelled spin column and centrifuged for 1 min at 12000 rpm. Collection tubes were discarded and each spin column was placed into a new collection tube. 500 µl of WASH buffer AW2 was dispensed into each spin column containing the collection tube and centrifuged for 1 min at 12000 rpm (9000 x g). Collection tubes containing waste was discarded and each spin column was placed into a new collection tube and centrifuged at 14000 rpm (10500 x g) for 3 mins. The spin column was placed into a new microcentrifuge tube (1.5 mL) and 60 µl of RNase-free elution buffer was dispensed into each tube containing the spin column. It was then incubated at room temperature for 1 min and centrifuged for 1 min at 12000 rpm. The spin column were discarded

Real Time Polymerase Chain Reaction (Rt-PCR)

Procedure of One Step Rt-PCR

Primer design

In order to design specific qPCR primers specific to the quantification of Homo sapien BCL-2 genes, Homo sapien BCL-2 were downloaded from the NCBI website (National Center for Biotechnology Information) and multiple cluster analysis was performed to reveal the conserved regions. Homo sapien BCL-2 genes of accession number NM_000633.3 was used as a DNA reference sequence and used in the primer designing. https://www.idtdna.com/PrimerQuest/Home/Index site was accessed and sequence pasted in the sequence entry box and multiple intercalating dye PCR primers were generated. It is very necessary to ensure that the primers will have a perfect match, this will enhance primer annealing during PCR. To do this, primers must anneal to regions where the sequences are conserved. Each primer pair was then checked for specificity to be sensitive to only the genes of interest to which it was designed to detect and also ability to cut across all aligned genes then the best primer was selected and synthesized at Inqaba in South Africa.

RNA treatment

20 ng total RNA was then treated with NEB DNase 1 (M0303) to totally eliminate extracted DNA briefly, a mixture of 2µl of 10ng/ µl RNA, 10 µl DNase I Reaction Buffer (10X), 1 µl DNAse I (RNase-free) and up to 100 µl with Nuclease-free H_2O. The mixture was then incubated at 37°C for 10 minutes followed by adding 1 µl of 0.5 M EDTA (to a final concentration of 5 mM). Then Heat inactivated at 75°C for 10 minutes and stored in the -20°C till use.

Gene quantification

A volume of 20 µl reactions following manufacturer's instructions using Luna® Universal qPCR Master Mix Protocol (M3003) was used to detect the presence of miRNA genes in the extracted RNA. Expression of Actin gene was used as an internal control. Briefly, a mix of 10 µl Luna Universal qPCR Master Mix, 0.5 µl Forward primer (10 µM) 0.5 µl Reverse primer (10 µM) and 0.06 Reverse Transcriptase (Promega) made up to 18 µl with Nuclease-free Water to which 2 µl of the treated RNA Template was added. This was then ran with the profile Initial Denaturation 95°C for 60 seconds followed by 40-45 of Denaturation 95°C 15 seconds Extension and plate reading at 60°C for 30 seconds followed by a termination at 72ºC for 10 minutes. Amplification was conducted using the CFX96TM REAL TIME SYSTEM FROM BIO-RAD following manufacturer manual. One-Step RT-PCR: It is a type of RT – PCR where the reverse transcription and the amplification reactions occur in a single tube. All the required components are added in a single tube. First, reverse transcription occurs, forming cDNA, which is then amplified in a PCR process. Luna Universal qPCR Master Mix and other reaction components was thawed at room temperature, then placed on ice. After thawing completely, each component was mixed briefly by inversion, pipetting or gentle vortexing. The total volume for the appropriate number of reactions was determined, plus 10% overage and assay mix of all components was prepared except DNA template accordingly. Then it was mixed thoroughly but gently by vortexing. Liquid collected to the bottom of the tube by brief centrifugation. Aliquot Assay mix was aliquoted into qPCR tubes or plate. For best results, accurate and consistent pipetting, precise volume measurements, and the minimization of bubbles were ensured. DNA templates was added to qPCR tubes or plate. Tubes were sealed with flat optically transparent caps, and plates sealed with optically transparent film. To prevent artifacts caused by evaporation, care was taken to have plate edges and corners sealed properly. Tubes or plates were spun briefly to remove bubbles and collect liquid (1 minute at 2500 – 3000 rpm). Real – time instrument was programmed with indicated thermocycling protocol. It was ensured that a "plate read" is included at the end of the extension step. The STBR or SYBR/FAM scan mode setting on the real-time instrument was used, along with the "Fast" cycling profile. During the RT phase, conducted at 42°C for 30 minutes, the Promega reverse transcriptase (RT)

enzyme converted the extracted RNA into cDNA. Data was analyzed according to real – time instrument manufacturer instructions.

Statistical analysis

The collected data was cleaned, coded, summarized, and checked for accuracy, consistency and completeness. The data is carefully entered into the Statistical Package for social science (SPSS IBM version 22) statistical software and analyzed using descriptive statistics such as mean, median, mode, standard deviation and bivariant analysis with T-test and cross tabulation/ correlation analysis with bar chat and histograms.

Results

In Figure 1, a Mann-Whitney U test was performed to compare the quantified expression of the BCL-2 gene between CML patients and healthy control individuals for prognosis. There was a statically significant difference in BCL gene expression across the two groups ($U = 1677.50; Z = 2.947; p < 0.05$). The lower the Ct values, the more the gene PCR product, the more the gene expression. The CML patients shows higher Bcl-2 expression from -0.7 to + 5.0 at the frequency of 5 to 10 compared to control subjects with peak gene expression from 0.00 to 10.0 at the frequency 5 to 9.

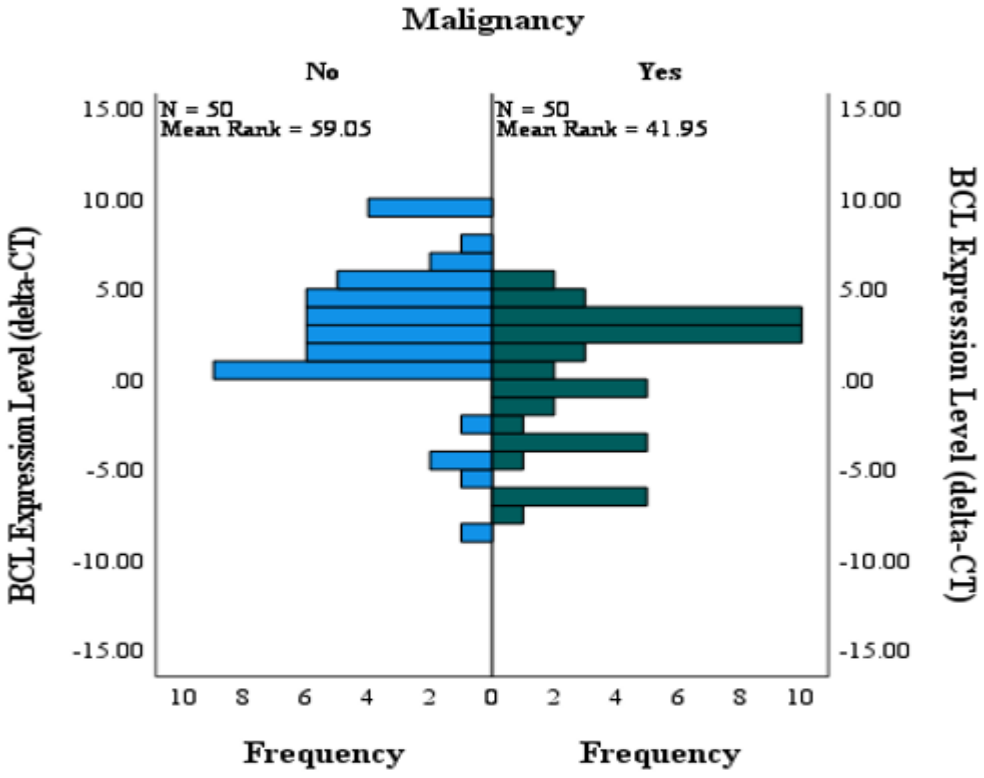


Figure 1. Comparing the quantified expression of the BCL-2 gene between CML patients and healthy control individuals for prognosis.

KEY: Threshold cycle (deltaCT): negative (-ve) values means Bcl-2 gene overexpression positive(+ve) value means under expression.

Figure 2 demonstrates the association between BCL-2 gene expression and WBC counts in CML patients. Among the 40% of patients showing BCL-2 overexpression, most—15 patients (30%)—presented with leukocytosis (>12.0–450.0 × 10^9/L), while 5 patients (10%) maintained WBC values within the normal range (3.0–12.0 × 10^9/L). In contrast, among the 60% of patients

without detectable BCL-2 expression, the majority—29 patients (58%)—had normal WBC levels, and only 1 patient (2%) exhibited leukopenia (<3.0 × 10⁹/L). This pattern suggests that BCL-2 overexpression is more frequently associated with elevated WBC counts in CML.

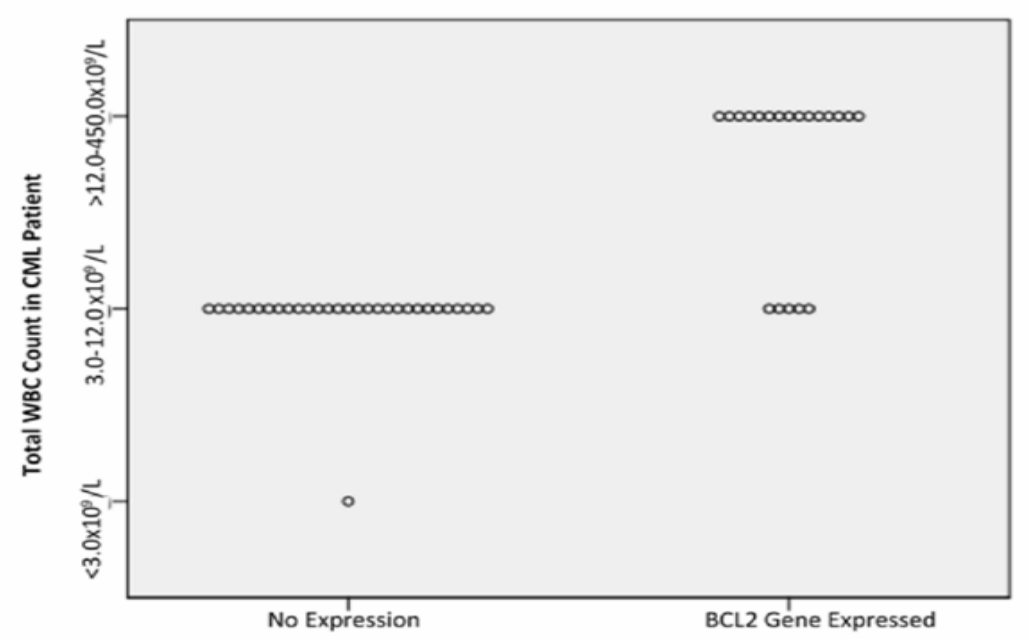
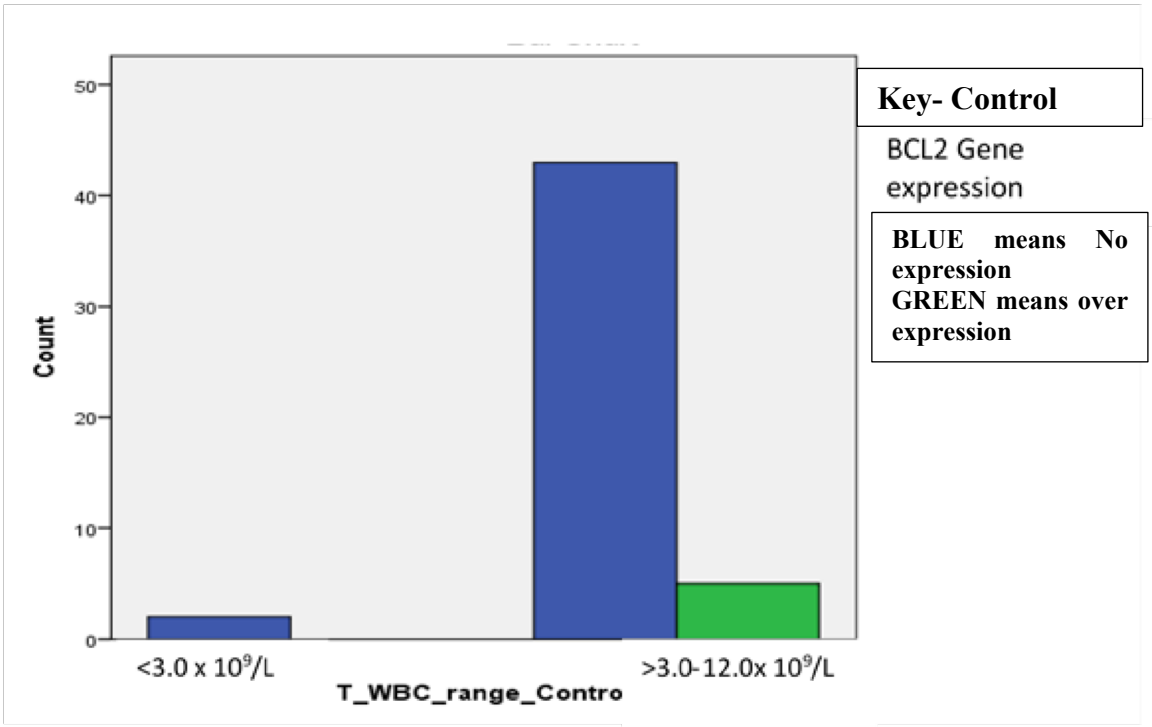


Figure 2. Bcl-2 gene expression in CML patients in relation to WBCs count (P=0.00)

In Figure 3, the relationship between BCL-2 gene expression and WBC count among the control subjects is presented. In this study, 43 (86%) of the 50 control subjects with no detectable BCL-2 expression had WBC counts within the normal reference range (3.0–12.0 × 10⁹/L). Additionally, 5 (10%) of the controls with BCL-2 overexpression also had WBC counts within the normal range, while 2 (4%) of those with no BCL-2 expression exhibited leukopenia (<3.0 × 10⁹/L).



Control Twbcs range (Total White blood cells range)

Figure 3. The relationship between BCL2 gene expression and WBC in control subjects

Table 1 highlights the haematological characteristics of participants in relation to BCL-2 expression. A notable association was identified between BCL-2 expression and packed cell volume (PCV) among CML patients. Among those with BCL-2 overexpression (40%), over half—12 patients (24%)—had reduced PCV values (<35%). Among the 60% of CML patients with no BCL-2 expression, 22 (44%), over half fell within the normal PCV range. In comparison, among the control participants, the 5 participants (10%) exhibiting BCL-2 overexpression included 4 (8%) with a normal PCV. Among controls without expression (90%), 30 (60%), over half maintained normal PCV levels. Regarding platelet profiles in CML patients, BCL-2 overexpression was associated with a broader distribution of platelet abnormalities. Of the 40% with overexpression, 11 (22%) had normal counts. Among the 60% without BCL-2 expression, over half-19 (38%) had normal counts. In the control group, the majority of those without expression (90%) had normal platelet levels (80%). Notably, all 5 controls with BCL-2 overexpression had platelet counts within the normal reference range. Other haematological parameters in relation to BCL-2 expression:

Neutrophils: Among BCL-2–expressing participants, 20% out of 40% CML patients showed neutrophilia. All control participants with BCL-2 expression (10%) had normal neutrophil counts. Among those without BCL-2 expression, 38% out of 60% CML patients exhibited normal counts whereas controls had 78% normal counts. Lymphocytes: In BCL-2–expressing participants, CML patients exhibited 24% lymphopenia. All controls with BCL-2 expression (10%) had lymphocytosis. Among participants without BCL-2 expression, CML patients had 52% lymphocytosis while the control group had 54% lymphocytosis. Red cell indices in relation to BCL-2 expression: Among 40% CML patients with BCL-2 expression: MCV: 28% normal. MCH: 16% normal, 16% low. MCHC: 30% normal. Among 60% CML patients without BCL-2 expression: MCV: 52% normal. MCH: 40% normal. MCHC: 56% normal. Among control participants: MCV: 44% normal, 46% low. MCH: 38% normal, 50% low. MCHC: 80% normal.

Table 1. Haematological profiles among the Bcl-2 overexpressed and Non-expressed participants

Profiles	Expression				No Expression				P-Value
	CML n=20 (40%) mean		Control n=5 (10%) mean		CML n=30 (60%) mean		Control n=45 (90%) mean		
Neutrophil									
Low	1(2.0%)	39.2	-	-	11(22.0%)	35.0±5.4	6(12.0%)	36.0±4.1	0.047
Normal	9(18.0%)	59.54±12.5	5(10.0%)	46.06±1.91	19(38.0%)	52.4±6.3	39(78.0%)	51.3±8.0	
High	10(20.0%)	80.12±4.1	-	-	-	-	-	-	
Lymphocyte									
Low	12(24.0%)	11.61±3.84	-	-	-	-	1(2.0%)	19.6	0.000
Normal	3(6.0%)	26.36±2.80	-	-	9(18.0%)	34.02±3.77	17(38.0%)	34.72±4.25	
High	5(10.0%)	44.0±4.66	5(10.0%)	46.38±2.72	21(42.0%)	51.33±7.74	27(54.0%)	47.27±4.63	
Mixed Cells									
Low	-	-	-	-	-	-	-	-	
Normal	14(28.0%)	7.70±1.89	5(10.0%)	8.22±1.43	26(52.0%)	7.24±1.20	34(68.0%)	7.18±1.53	0.001
High	6(12.0%)	12.88±1.84	-	-	4(8.0%)	11.32±0.72	11(22.0%)	12.36±2.31	
MCV									
Microcytic	6(12.0%)	72.13±7.72	3(6.0%)	64.86±12.32	2(4.0%)	48.50±19.65	23(46.0%)	72.17±6.68	0.000
Normocytic	14(28.0%)	86.62±4.92	2(4.0%)	84.50±3.25	26(52.0%)	88.75±4.59	22(44.0%)	86.72±4.01	
Macrocytic	-	-	-	-	2(4.0%)	102.75±2.47	-	-	
MCH									
Hypochromic	8(16.0%)	24.07±3.22	3(6.0%)	20.30±5.07	6(12.0%)	24.55±1.72	25(50.0%)	23.68±2.81	0.012
Normochromic	8(16.0%)	29.47±1.32	2(4.0%)	29.30±0.98	20(40.0%)	29.76±1.11	19(38.0%)	29.21±0.96	
Hyperchromic	4(8.0%)	41.10±10.18	-	-	4(8.0%)	33.17±1.21	1(2.0%)	33.2	
MCHC									
Hypochromasia	1(2.0%)	29.0	1(2.0%)	28.9	2(4.0%)	30.65±0.07	5(10.0%)	30.06±0.64	0.001
Normochromasia	15(30.0%)	32.98±0.53	4(8.0%)	33.40±1.67	28(56.0%)	33.49±0.75	40(80.0%)	33.22±0.87	
Hyperchromasia	4(8.0%)	43.92±8.19	-	-	-	-	-	-	
PCV									
<35%	12(24.0%)	30.35±3.74	2(4.0%)	32.90±0.42	8(16.0%)	29.36±5.16	14(28.0%)	32.78±2.18	0.011
35-54%	7(14.0%)	40.75±5.85	3(6.0%)	41.13±3.55	22(44.0%)	41.57±3.65	30(60.0%)	41.31±4.37	
>54%	1(2.0%)	66.30	-	-	-	-	1(2.0%)	54.50	
PLATELET									
Thrombocytopenia	2(4.0%)	69.50±13.43	-	-	10(20.0%)	119.2±22.57	3(6.0%)	132.0±13.45	0.000
Normal	11(22.0%)	248.91±77.2	5(10.0%)	234.8±86.3	19(38.0%)	214.6±54.70	40(80.0%)	224.9±49.66	
Thrombocytosis	7(14.0%)	644.0±129.5	-	-	1(2.0%)	490	2(4.0%)	453.0±7.07	

Key: haematology parameters normal reference range:
Neutrophil: 40-75%, Lymphocyte: 21-40%, Platelets: 150,000- 400,000/ml,
Mixed cells: 2-10%. Red cell Indices: MCV=80-98fl, MCH=27-32pg, MCHC=31.5-36.0g/dl

Discussion

The socio-demographic profile of the study participants indicated that the majority of CML patients 42% were aged between 41 and 60 years, with very few participants in the 81–90 age range. Females accounted for more than half of the CML group 58%. Most participants 86% had attained tertiary education, while only 6% had no formal education. In the control group, the predominant age range was 21–40 years, with males representing the larger proportion 60%, and approximately half 48% having completed secondary education.

In this study, participant age was not included as a variable. However, previous work by Liu *et al*., (2019) reported that BCL-2 expression exhibits a complex age-related pattern, decreasing in certain tissues while increasing or remaining unchanged in others [27]. Gender was likewise not considered, as BCL-2 plays a fundamental role in hematopoiesis by supporting the survival of hematopoietic cells[4]. Although chronic myeloid leukemia (CML) may occur at any age, it is rare in children[28].

All CML participants in this study were receiving CML-specific therapy. Among them, 16% initiated treatment in 2008, 14% in 2013, 18% in 2017, and 26% began therapy in 2021, with all continuing treatment through the study period in 2024. Treatment history was not analyzed as a variable because findings by Lee et al. (2021) indicate that the gene of interest significantly upregulates BCL-2 expression in CML patients, independent of disease duration [29]. Furthermore, BCL-2 overexpression is known to block the intrinsic apoptotic pathway activated by tyrosine kinase inhibitors (TKIs) by suppressing pro-apoptotic proteins[30]. This mechanism contributes to TKI resistance and the prolonged survival of CML stem cells[31].

Among the 40% of CML patients who demonstrated BCL-2 overexpression, 14% had normal packed cell volume (PCV), 24% had reduced PCV, and 2% had elevated PCV. Thus, nearly one-quarter of the overexpression group presented with anemia. In contrast, among the 60% of patients without BCL-2 expression, 16% had low PCV while 44% had normal PCV. This indicates that more than half of the patients with BCL-2 overexpression were anemic, whereas over half of those without BCL-2 expression maintained normal PCV values, a difference that was statistically significant ($P < 0.05$).

Similarly, within the subgroup of CML patients exhibiting BCL-2 overexpression, 30% presented with leukocytosis and 10% had normal white blood cell (WBC) counts. Among the 60% of patients without BCL-2 expression, 58% had normal WBC counts, while 2% showed leukopenia.

Conclusions

The overexpression of BCL-2 observed in this study is characteristic of CML and its association with anaemia, leukocytosis and neutrophilia proves that Bcl-2 expression level is a marker for the role of BCL-2 gene in the incidence of CML disease and can be used in the stratification and evaluation of hematologic malignant (CML) disease. Targeting a driver of haematologic malignancies (H.M.) is an effective approach to identify possible prognosis for these diseases and aid in the BCL-2 therapy.

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QUESTIONNAIRE/CONSENT FORM

ASSESSMENT OF BCL-2 GENE MUTATION BY ITS EXPRESSION LEVEL IN HAEMATOLOGIC MALIGNANCIES IN ILE IFE, OSUN STATE

Good day, Sir/Ma. This questionnaire is being administered to you to determine the importance of, ASSESSMENT OF BCL-2 GENE MUTATION BY ITS EXPRESSION LEVEL IN HAEMATOLOGIC MALIGNANCIES (CML) IN ILE IFE, OSUN STATE Please respond honestly to the question below by ticking the appropriate response (√). The confidentiality of your response is guaranteed.

DATE…… I.D NUMBER……..

SECTION A (SOCIO-DEMOGRAPHIC CHARACTERISTICS)

Age (years)…….. Gender: (Male) (Female)

SECTION B (MEDICAL HISTORY)

When were you diagnosed with CML? ……or Any other types……Specify…….

Are you using any drugs currently? Yes ( ) No ( )

If Yes please state the name of the drug …….

When did you commence the use of this drug?.....

Are you being treated for any other medical condition? Yes ( ) No ( )

If yes please explain briefly_____

_____

Are you aware of BCL2 mutation? (Yes) (No)

If yes, Has the test been conducted on you before? (Yes) (No)

If yes, kindly give a short detail of the test's outcome_____

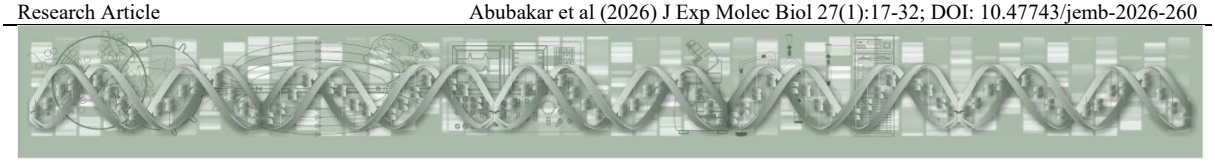
_____

__

Thanks for your co-operation.

Socio-demographic characteristics of CML patients and Control subjects

	Chronic Myeloid Leukemia Patients		Control Group	
Variable	**Frequency (N=50)**	**Percentage**	**Frequency (N=50)**	**Percentage**
Age				
1-20	3	6.0	3	6.0
21-40	17	34.0	28	56.0
41-60	21	42.0	19	38.0
61-80	8	16.0	0	0.0
81-100	1	2.0	0	0.0
Gender				
Male	21	42.0	30	60.0
Female	29	58.0	20	40.0
Education				
None	3	6.0	0	0.0
Primary	2	4.0	4	8.0
Secondary	2	4.0	24	48.0
Tertiary	43	86.0	22	44.0
Year of onset of disease				
2008-2012	8	16.0	Not Applicable	
2013-2016	7	14.0		
2017-2020	9	18.0		
2021-2024	26	52.0		
Year of Placement on Treatment	8	16.0	Not Applicable	
2008-2012	7	14.0		
2013-2016	9	18.0		
2017-2020	26	52.0		
2021-2024				



PHYTOCHEMICALS, NUTRITIONAL AND ANTI-NUTRITIONAL COMPOSITION OF ETHANOL LEAVES EXTRACT OF *ELEUSINE INDICA*

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Abstract

Eleusine indica has been consumed for nutrition and management of many diseases including epilepsy, diarrhea, infections, dysentery, influenza, and hypertension. This study aimed at evaluating the phytochemicals, nutrients and anti-nutrients composition of ethanol leaves extract of *E. indica*. Phytochemicals, proximate, and anti-nutrients content of the extract were determined using AOAC method. The levels of minerals in the extract were estimated using atomic absorption spectrophotometeric method (AAS) and flame photometeric technique. The extract displayed significant ($p < 0.05$) amount of steroids (78.75 %), flavonoids (52.10 **%**), terpenoids (44.14 %), tannins (40.25 %), alkaloids (26.13 %), saponin (34.76 %) and phenols (36.00 %). The significant ($p < 0.05$) amount of crude protein, crude fat, crude fiber, ash, moisture content, and carbohydrate observed in the extract was 7.43 %, 6.17 %, 24.60 %, 15.40 %, 13.50 %, and 35.50 %, respectively. The extract contains significant ($p < 0.05$) amount of magnesium (9.33 mg/100g), sodium (2.66 mg/100g), potassium (3.00 mg/100g), calcium (7.33 mg/100g), iron (4.68 mg/100g), zinc (8.89 mg/100g), and copper (5.48 mg/100g). However, low level of oxalate (2.36 mg/100g), alkaloid (1.42 mg/100g), saponin (3.27 mg/100g), phytate (3.30 mg/100g), and tannin (1.70 mg/100g) was detected in the extract. The ethanol leaves extract of *Eleusine indica* displayed significant amount of phytochemicals, proximate nutrients, and minerals suggesting their nutritional and therapeutic properties.

Keywords: Anti-nutrients, *Eleusine indica*, Minerals, Nutrients, Phytochemicals

Introduction

Demand for food and medicine, has been increasing due to the rapid population of people in the world. Inadequate of nutrients rich foods remains a major public health challenge causing a number of deaths especially in African countries. Insufficient quality plants-based foods cause adverse health effects, economic, and environmental impacts. About 45 % of all deaths among children under the age of five is associated with insufficient nutrients rich foods (UNICEF 2019). In 2024, report showed that 2.6 billion people could not afford a healthy diet and about 673 million people in the world that is approximately 8.3 % of the world population were suffered with excessive hunger (UNICEF 2025). Nutrients or foods rich nutrients are required for several physiological and biochemical processes. Foods rich nutrients aid physiological functions required for developing human body system (Onireti and Ikujenlola 2020). Nutrients enhance growth and development of tissues and regulation of metabolic activities in the body (Alexander and Eli 2025). More than ten million people are malnourishing due to excessive

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hunger worldwide (WFP 2025). Malnutrition results due to lack or inadequate of micronutrient and macronutrient rich diets (WHO 2024). It is predicted that by 2050 the population of people in the world will approximately reach 8 billion and demand for foods will be major concern (Saerens et al. 2021).

Natural products particularly plants and herbs have been consumed for nutrition and remedies. About eighty percent of the world's population depends on plants for foods and remedies (Khan and Ahmad 2019, Abubakar et al. 2024, Abubakaret al. 2025). Plant based foods enhance dietary diversity and nutritional intake in local and resource limited communities (Rumicha et al. 2025). Plants and herbs displayed several nutritional and medicinal properties due to their nutrients contents and phytoconstituents (Abubakar et al. 2024, Abubakar et al. 2025). Plants and their products serve many biological functions and exhibited different pharmacological activities (Abubakar et al. 2025). Medicinal plants constitute different bioactive compounds that have important application in pharmaceutics and pharmaceutical industries for drug synthesis (Abubakar et al. 2022, Abubakaret al. 2025).

Phytochemicals are bioactive compounds produced in plants and characterized by low molecular weight (Chihomvu et al. 2024). They are secondary metabolites produce in plants where they serve significant roles including attracting pollinators and defending against herbivores (Thirumurugan et al. 2018, Chaachouay and Zidane 2023). Plants derived from phytochemicals exhibit protective effect against a number of diseases including cancer, coronary heart disease, neurological disorders, diabetes, hypertension, inflammation, infections, and gastrointestinal diseases (Prakash et al. 2012). They displayed a significant potential in the regulation of epigenome and produced very low toxic effect when consumed over a long period of time (Ramírez-Alarcón et al. 2021). Phytochemicals demonstrate significant pharmacological activities including antioxidant, anti-inflammatory, antimicrobial and anticancer activities (Rodríguez-Negrete et al. 2024, Janja 2025). Anti-nutritional factors are chemical compounds produced in food that reduce bioavailability of nutrients by decreasing the absorption of essential nutrients (Abubakar et al. 2022). Anti-nutrients produce adverse health effects on human and animal health (Abubakar et al. 2025). Anti-nutritional factors inhibit the utilization of nutrients, particularly proteins, vitamins, and minerals, thereby reducing nutritional benefits and value of foods (Fekadu et al. 2013, Tadessea et al. 2025).

Eleusine indica, commonly known as Goose grass is a diploid plant that belongs to the family Poaceae (Ettebong and Bassey 2017). The plant is widely available in tropical and subtropical countries. *E. indica* has been used in traditional treatment of many disorders including microbial infections, sprained muscle, coughing blood, and insects poisoning (Zakri et al. 2021). Fresh leaves of the plant have been used in treatment of diarrhea, dysentery, epilepsy, influenza, hypertension, and oliguria (Sagnia et al. 2014, Iberahim et al. 2015, Tutor and Chichioco-Hernandez 2018). The roots of the plant have been used in the local treatment of snake bites (Upasani et al. 2018). Aqueous extract made from infusing aerial parts of *E. indica* has been used for treatment of flu-related symptoms (Piah 2020). *E. indica* demonstrates significant anti-inflammatory, antioxidant, antimicrobial, hepatoprotective, anti-plasmodial, anti-diabetic, and anti-cancer activities (Nas et al. 2020, Shatakshi et al. 2025). In Nigeria, *E. indica* is locally called Gbegi or Gbaji in Yoruba, and Ciyawan daji in Hausa.The plant has been used in many local communities in Nigeria for the treatment of malaria, diabetes, gastrointestinal disorders and microbial infections.

Materials and Methods

Chemicals and Reagents

All the experiments were conducted using the analytical graded chemicals and reagents manufactured by Sigma-Aldrich based in St. Louis, MO, USA and Guangdong Chemical Reagent Engineering based in Guangdong, China.

Plant Sample

Fresh leaves of *Eleusine indica* were collected from *Gwadangwaji* village in Birnin Kebbi, Kebbi State, Nigeria. The samples were identified and transported to Biochemistry Laboratory, Department of Science Laboratory Technology, Waziri Umaru Federal Polytechnic Birnin Kebbi, Kebbi State, Nigeria for analyses.

Plant Extract

The plant leaves were washed thoroughly with distilled water and shed dried for seven days at room temperature. The dried samples were grounded to fine powder using motar and pestle. Extraction of the plant sample was done according to the method described by Abubakar et al. (2021). The powder sample (500 g) was extracted in 1 L of ethanol for 72 hours with shaken at one hour interval. The extract was filtered through a Whatman filter paper No 1 and then concentrated to dryness. The extract was weighed (154.7 g) and the percentage yield (30.94 %) was calculated and recorded. The extract was stored in the refrigerator at 4 ^{o}C for further analysis.

Qualitative Phytochemicals Analysis

The qualitative and quantitative determination phytochemicals of composition of the extract was conducted according to the standard methods described by AOAC (1999), Trease and Evans (1989), Harborne (1973), Mosa et al. (2012), Abubakar et al. (2020), and Ibrahim et al. (2024).

Alkaloids

The presence of alkaloids in the ethanol leaves extract of *E. indica* was detected using Wagner's test as described by Trease and Evans (1989) and Abubakar et al. (2020). The extract was treated with 3 mL of 1 % HCl solution in a test tube which was heated for 20 minutes and then allowed to cool at room temperature. The content was treated with 1 mL of Wagner’s reagent in drops resulting to the formation of reddish-brown precipitate indicating that alkaloids were present in the extract.

Flavonoids

Flavonoids present in the extract were identified by NaOH test according to the method described by Mosa et al. (2012) and Ibrahim et al. (2024). Ten percent sodium hydroxide solution (1 mL) was added into a test tube containing 3 mL of the extract. Flavonoids in the extract were detected by formation of deep yellow precipitate which upon addition of HCl became colourless.

Tannins

Ferric chloride test was used for qualitative determination of tannins in the extract according to the method of Trease and Evans (1989) and Ibrahim et al. (2024). The extract (1 mL) was treated with 2 mL of 5 % $FeCl_2$ solution. The formation of black or blue-green colour indicated the present of tannins in the extract.

Saponins

Saponins in the extract were qualitatively estimated using Froth test as described by Mosa et al. (2012) and Abubakar et al. (2022). The extract (3 mL) was transferred into a test tube followed by addition of 3 mL of distilled water. The contents in the test tube were shaken for 30 sec and settled for 30 minutes. The presence of saponins in the extract was observed by formation of stable persistent froth.

Steroids

The identification test for steroid in the extract was carried out using the method of Trease and Evans (1989) and Ibrahim et al. (2024). The extract (500 µL) was transferred into the test tube followed by addition of chloroform (5 mL) and H_2SO_4 (5 mL) solution. The violet colour later blue-green indicating that steroids were present in the extract.

Terpenoids

The screening of terpenoids in the extract was done according to the method described by Trease and Evans (1989) and Abubakar et al. (2020). The ethanol (1 mL) and acetic anhydride (1 mL) was added into the test tube containing extract. The mixture was treated with 10 mL of H_2SO_4 solution. The present of terpenoids was detected by development of pink color.

Test for Phenols

The extract was screen for the presence of phenols employing the method of Trease and Evans (1989) and Abubakar et al. (2022). Five miles of the extract was transferred in a test tube followed by addition of ethanol (5 mL) and ferric chloride (5 mL) resulting to the formation of ink blue color indicating the presence of phenols.

Quantitative Estimation of Phytochemicals

Alkaloids

The amount of alkaloids in the extract was estimated using according to the method described by Trease and Evans (1989) and Ibrahim et al. (2024). The dried extract (5 g) was treated with 100 mL of methanol and then evaporated in rotary evaporator. Twenty miles of 2 mM H_2SO_4 was added into the content, shaked vigorously, and then treated with ether. The upper phase of the solution was treated with NH_3 solution and followed by extraction with chloroform. The extract containing alkaloids was dried in oven and then weighed. The alkaloids content was obtained using the formula below:

$$\text{Alkaloids Content (\%)} = \frac{\text{Weight of alkaloids residue}}{\text{Weight of extract}} \text{ x } 100$$

Flavonoids

Flavonoids present in the extract were quantitatively determined using the method of Harborne (1973) and Ibrahim et al. (2024). The extract (5 mg) was treated with 50 mL of 2M HCl solution followed by boiling for half hour. The mixture was allowed to cool and then filtered using Whatman filter paper. The ethylacetate solution (50 mL) was added to the filtrated. The mixture was filtered and then concentrated to dryness. The flavonoids residue was weighed and the flavonoids content was calculated using the following equation:

$$\text{Flavonoids Content (\%)} = \frac{\text{Weight of flavonoids residue}}{\text{Weight of extract}} \text{ x } 100$$

Tannins

Spectrophotometric method described by AOAC (1999) was employed for quantitative analysis of tannins in the extract. Ten milligram of tannic acid was dissolved in 100 mL of distilled water. The solution was used for preparation of tannic acid standards (0 – 2.5 mL) in 25 mL volumetric flasks. The extract (1 g) was boiled in 80 mL of distilled water for half hour. The contents were treated with 2.5 mL of Folin-Ciocalteu reagent and 1.25 mL of sodium carbonate solution and then incubated at room temperature for half hour. The absorbance was read spectrophotometerically at 760 nm wavelength. The tannic acid standard curve was constructed and the tannin content in the extract was obtained from the standard curve.

Saponins

Saponins present in the extract were quantitatively determined using the method of El-Olemyl et al. (1994) and Ibrahim et al. (2024). The extract (5 g) was treated with 150 mL of 50 % ethanol, boiled for half hour and then filtered using Whatman filter paper. The filtrate was treated with 1 g of charcoal, boiled for half hour, filtered and then cooled at room temperature. The filtrate was treated with 150 mL of acetone, filtered and the residue was immediately taken into the desiccator containing anhydrous $CaCl_2$ solution. The solution was dried in oven, weighed and the saponins content in was calculated using the equation below:

$$\text{Saponins Content (\%)} = \frac{\text{Weight of saponins residue}}{\text{Weight of extract}} \text{X } 100$$

Steroids

The steroids content of the extract was evaluated using the method of Trease and Evans (1989) and Ibrahim et al. (2024). The extract (1 mL) was transferred into a test tube followed by addition of 2 mL of H_2SO_4 and $FeCl_2$ solution. The mixture was treated with 2 mL of potassium hexacynoferrate (III) solution and then incubated at 70 °C for half hour. The absorbance of the extract was measured spectrophotometerically at 780 nm wavelength. The steroids content was calculated using the following equation:

Steroids Content (%) = Absorbance of extract × 100

Proximate Analysis

Moisture

Oven-drying method was employed for determination of moisture content of the extract as described by Miroslav and Vladimir (1999) and Aliyu et al. (2026). A crucible was clean, dried, weighed (W_1) and then heated. One gram of the extract (W_2) was transferred into the weighed preheated crucible. The crucible containing the extract was heated in an oven at 125 °C for 3 hours until a consistent weight was obtained. The content was allowed to cool and then weighed (W_3). The moisture content of the extract was calculated using the following equation:

$$\text{Moisture content (\%)} = \frac{W3 - W1}{W2} \text{ x } 100$$

Crude protein

The crude protein content of the extract was estimated using Kjeldahl method as described by Chen et al. (2006). The dried extract (2 g) was digested in a digesting tube containing mixture of sulfuric acid, potassium sulfate, copper sulfate, and selenium for four hours. The digested material was treated with boric acid and sodium hydroxide for nitrogen distillation. The distillate material was titrated with 0.05 N H_2SO_4 solution. The crude protein content was obtained using the following equation:

$$\text{Protein (\%)} = \frac{\text{Titre value x 14 x Volume x Normality x 6.25}}{\text{Conc. of solution x 1000 x weight of sample}} \text{ x } 100$$

Ash

The ash content of the extract was estimated using the method described by AOAC (2010) and Aliyu et al. (2025). A clean empty crucible was weighed (W_1) and then heated in an oven at 105 ˚C for 1 hour. One gram of the extract (W_2) was taken to the crucible and then heated in a muffle furnace at 600 ˚C for 1 hour. The crucible was allowed to cool at room temperature and then weighed (W_3). The ash content was calculated using the formula below:

$$\text{Ash content (\%)} = \frac{W3 - W1}{W2} \text{ x } 100$$

Crude Fiber

The crude fiber content of the extract was determined by weight difference as described by Olszewska et al. (2001). The extract (1 g) was treated with 100 mL of 1.25 % H_2SO_4 and then with 100 mL of 1.25 % NaOH solution. The mixture was filtered, washed with distilled water, dried in oven and then ashed in the furnace at 550 ºC for 60 minutes. The fiber content was calculated using the following formula:

$$\text{Fiber (\%)} = \frac{\text{Weight of crucible} + \text{Weight of sample} - \text{Weight of crucible}}{\text{Weight of sample}} \text{ x } 100$$

Crude lipids

The dried extract was weighed (4 g) and transferred into a separate extraction thimble and extracted with 200 mL of petroleum ether at temperature 60 – 80 ºC for 8 hours. The extract was dried in oven at 103 ºC to evaporate the solvent. The extract was allowed to cool and then weighed. The fat content of the extract was calculated using the formula below:

$$\text{Fat (\%)} = \frac{\text{Weight of thimble before extraction} - \text{Weight of thimble after extraction}}{\text{Weight of sample}} \text{ x } 100$$

Carbohydrate

The carbohydrate content of the extract was determined by difference method using the equation below:

Carbohydrates = 100 – (Protein + Fat + Ash + Moisture)

Estimation of Minerals Content

The quantitative estimation of level of magnesium, calcium, iron, zinc, and copper in the extract was performed using atomic absorption spectrophotometeric (AAS) method as described by AOAC (1990) and Abubakar et al. (2025). Flame photometeric technique was employed for the determination of concentration of sodium and potassium in the extract using the method of AOAC (1990; 2005). The plant sample was burn in a muffle furnace and then digested using nitric acid. The digested sample was extracted with methanol and then analyzed by atomic absorption spectrophotometeric and/or flame photometeric technique.

Determination of Anti-nutrients Contents

The quantitative estimation of alkaloid, saponin, oxalate, phytate, and tannin level in the extract using the method of Harborne (1984), and Abubakar et al. (2025).

Oxalate

Titration technique was employed for estimation of oxalate in the extract as described by Gupta et al. (2005) and Onwuka (2005). Two gram of the extract was dissolved in 190 mL of deionized followed by addition of 10 mL of 6M HCl. The solution was digested at 100 ^{o}C for 60 minutes, cooled and filtered using filter paper. The filtrate was precipitated with NH_4OH and then treated with 10 mL of 20 % H_2SO_4 solution. The solution was titrated with 0.05 M potassium permanganate. The oxalate content was obtained using the following formula:

$$\text{Oxalate (\%)} = \frac{\text{T x Vme x DF x 105}}{\text{ME x MF}} \text{ x } 100$$

Where; T is titre value of $KMnO_7$, Vme is volume-mass equivalent (1 mL of $KMnO_7$ = 0.00225), DF is dilution factor, ME is molar equivalent of $KMnO_7$, MF is mass of the sample.

Tannins

Spectrophotometric method described by AOAC (1999) was employed for quantitative analysis of tannins in the extract. Ten milligram of tannic acid was dissolved in 100 mL of distilled water. The solution was used for preparation of tannic acid standards (0 – 2.5 mL) in 25 mL volumetric flasks. The extract (1 g) was boiled in 80 mL of distilled water for half hour. The contents were treated with 2.5 mL of Folin-Ciocalteu reagent and 1.25 mL of sodium carbonate solution and then incubated at room temperature for half hour. The absorbance was read spectrophotometerically at 760 nm wavelength. The tannic acid standard curve was constructed and the tannin content in the extract was obtained from the standard curve.

Alkaloids

The amount of alkaloids in the extract was estimated using according to the method described by Trease and Evans (1989) and Ibrahim et al. (2024). The dried extract (5 g) was treated with 100 mL of methanol and then evaporated in rotary evaporator. Twenty miles of 2 mM H_2SO_4 was added into the content, shaked vigorously, and then treated with ether. The upper phase of the solution was treated with NH_3 solution and followed by extraction with chloroform. The extract containing alkaloids was dried in oven and then weighed. The alkaloids content was obtained using the formula below:

$$\text{Alkaloids Content (\%)} = \frac{\text{Weight of alkaloids residue}}{\text{Weight of extract}} \text{ x } 100$$

Phytate

The phytate content of the extract was estimated using the method of Lucas and Markakas (1975) and Reddy and Love (1999). Two gram of the extract was treated with 100 mL of 2%

HCl and then filtered using Whatman No. 1 filter paper. The filtrate (50 mL) was treated with 10 mL of distilled water to adjust the pH of the solution followed by addition of 10 mL of 0.3 % ammonium thiocyanate solution. The solution was titrated with standard Iron II Chloride solution containing 0.00195 g Iron/mL until a yellow colour persisting up to five minutes. The phytate content was obtained using the following equation:

Phytate (%) = Titre value × 0.00195 g × 1.19 × 100

Saponins

Saponins present in the extract were quantitatively determined using the method of El-Olemyl et al. (1994) and Ibrahim et al. (2024). The extract (5 g) was treated with 150 mL of 50 % ethanol, boiled for half hour and then filtered using Whatman filter paper. The filtrate was treated with 1 g of charcoal, boiled for half hour, filtered and then cooled at room temperature. The filtrate was treated with 150 mL of acetone, filtered and the residue was immediately taken into the desiccator containing anhydrous $CaCl_2$ solution. The solution was dried in oven, weighed and the saponins content in was calculated using the equation below:

$$\text{Saponins Content (\%)} = \frac{\text{Weight of saponins residue}}{\text{Weight of extract}} \text{X } 100$$

Statistical Analysis

All the experimental analyses were repeated three times. The results were expressed as mean ± SEM. The data were analyzed using Statistical Package for Social Sciences (SPSS) Statistics version 22 software was used for the data analysis. One-way analysis of variance (ANOVA) was employed for determining the significant differences among the average values at95 % confidence level using Dunnett multiple comparison test. Two-tailed (p<0.05) were considered significant.

Results

Photochemicals Composition of Ethanol Leaves Extract of *Eleusine indica*

Table 1 shows the qualitative phytochemicals composition of ethanol leaves extract of *Eleusine indica.* The extract displayed the presence of high amount of flavonoids and steroids. Moderate amount of tannins and terpenoids were detected in the extract. Alkaloids, saponins, and phenols were slightly present in the extract (Table 1).

Table 1. Qualitative Phytochemicals Composition of Ethanol Leaves Extract of *Eleusine indica*

Phytochemical	Extract
Alkaloids	+
Flavonoids	+++
Saponins	+
Tannins	++
Phenols	+
Steroids	+++
Terpeniods	++

High (+++), Moderate (++), Fair (+)

Figure 1 shows the quantitative phytochemicals composition of ethanol leaves extract of *Eleusine indica*. The extract showed the presence of high significant ($p < 0.05$) amount of steroids (78.75 %) and flavonoids (52.10 **%**). The extract showed the presence of moderate significant ($p < 0.05$) amount of terpenoids (44.14 %) and tannins (40.25 %). Low amount of alkaloids (26.13 %), saponin (34.76%) and phenols (36.00 %) were found in extract (Figure 1).

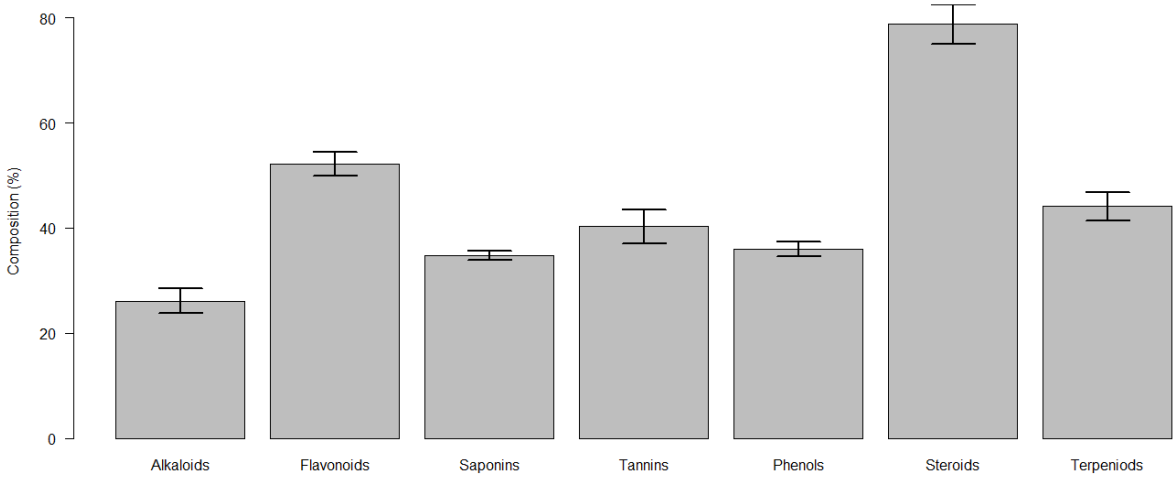

Figure 1. Quantitative Phytochemicals Composition of Ethanol Leaves Extract of *Eleusine indica*
Results are expressed as mean ± SEM (n = 3).

Proximate Composition of Ethanol Leaves Extract of *Eleusine indica*
The proximate composition of ethanol leaves extract of *Eleusine indica* is presented in Figure 2. The significant ($p < 0.05$) amount of crude protein, crude fat, crude fiber, ash, moisture content, and carbohydrate found in the extract was 7.43 %, 6.17 %, 24.60 %, 15.40 %, 13.50 %, and 35.50 %, respectively.

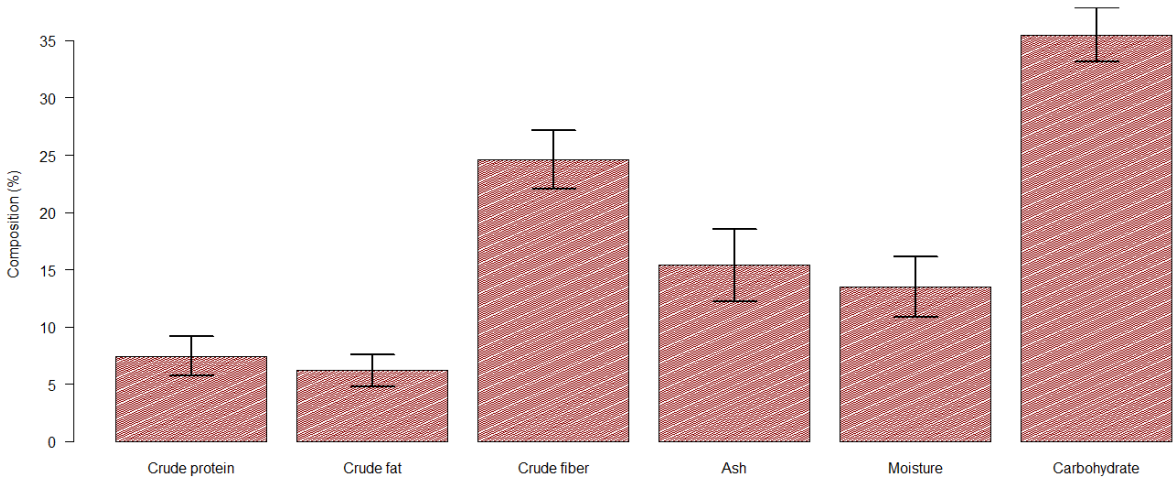

Figure2. Proximate Composition of Ethanol Leaves Extract of *Eleusine indica*
Values are expressed as mean ± SEM (n = 3).

Minerals Content of Ethanol Leaves Extract of *Eleusine indica*

Figure 3 shows the minerals content of ethanol leaves extract of *Eleusine indica*. The extract contains more significant ($p < 0.05$) amount of magnesium (9.33 mg/100g), sodium (2.66 mg/100g), potassium (3.00 mg/100g), calcium (7.33 mg/100g), iron (4.68 mg/100g), zinc (8.89 mg/100g), and copper (5.48mg/100g) (Figure 3).

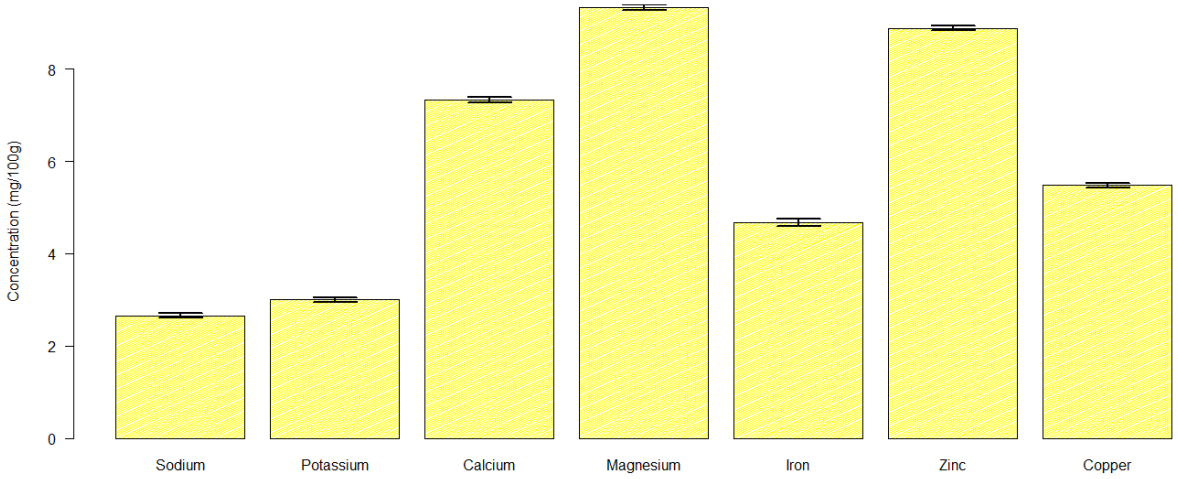

Figure 3. Minerals Content of Ethanol Leaves Extract of *Eleusine indica*
Data are expressed as mean ± SEM (n = 3).

Figure 4 shows the anti-nutrients content of ethanol leaves extract of *Eleusine indica*. The result showed the level of alkaloid, saponin, oxalate, phytate, and tannin in the extract was 1.42 mg/100g, 3.27 mg/100g, 2.36 mg/100g, 3.30 mg/100g, and 1.70 mg/100g, respectively.

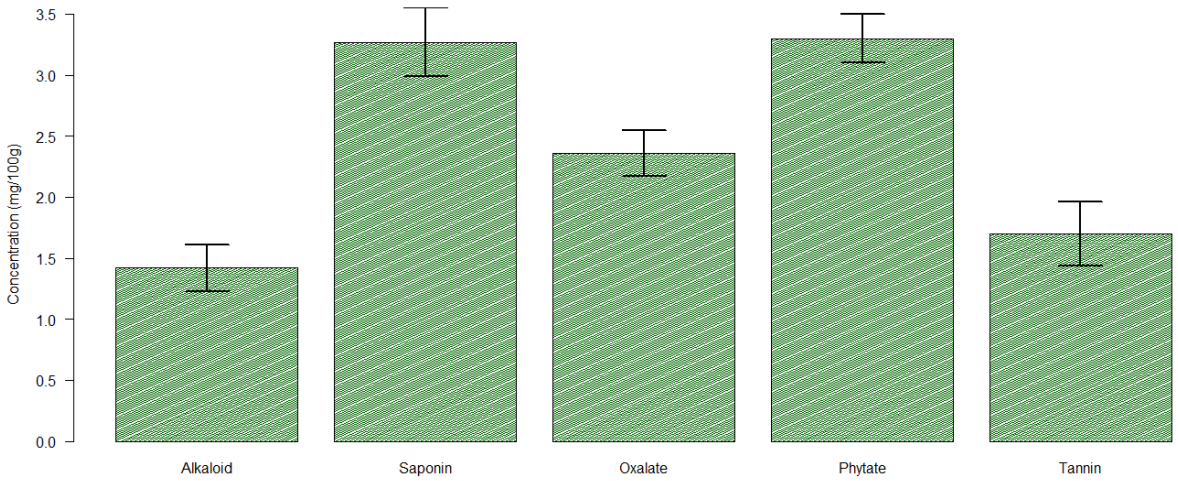

Figure 4. Anti-nutrients Content of Ethanol Leaves Extract of *Eleusine indica*
Results are given as mean ± SEM (n = 3).

Discussion

In this study, the ethanol leaves extract of *Eleusine indica* contain significant amount ofsteroids, flavonoids, tannins, alkaloids, saponins, and phenols. Result of this study is in agreement with

the result of similar study which revealed the presence of flavonoids, alkaloids, phenolics, terpenoids, saponins, and glycosides in *E. indica* (Shatakshi et al. 2025). This finding agrees with the result of the relevant study which showed that *E. indica* leaves contain significant amount of the phytochemicals alkaloids, flavonoids, steroids, glycosides, and saponins (Jone et al. 2023). Relevant studies revealed that *E. indica* contains saponins which contributed to its medicinal properties (Dorota et al. 2017, Shatakshi et al. 2025). Similar finding showed that *Eleusine indica* contain various phytochemicals such as phenols, anthraquinones, triterpenes, steroids, tannins, flavonoids, and alkaloids (Ette et al. 2020).

Phytochemicals from different plants extracts displayed a number of pharmacological activities (Oghenejobo et al. 2017). *E. indica* contains different phytochemicals that are responsible for its pharmacological activities (Shatakshi et al. 2025). Tannins in plants extracts exhibited astringents properties, anti-ulcer and analgesic activities (Abubakar et al. 2022). Saponins isolated from the plants extracts have been used in pharmaceutical industries as emulsifiers, foaming agents, and adjuvants in vaccines (Timilsena et al. 2023). Saponins have been documented to possess antifungal, antibacterial, and anti-inflammatory activities (Shatakshi et al. 2025). Alkaloids from the plants extracts exhibited various pharmacological activities including antimicrobial and anti-inflammatory activities (Joanna 2019, Shatakshi et al. 2025). Alkaloids have potential for treating diseases associated with nervous system (Owheruo et al. 2018). Flavonoids have been reported with anti-inflammatory and anti-cancer activities (Mutha et al. 2021). Flavonoids exhibited significant antioxidant activities and used in neutralizing poisons in the body (Edo et al. 2023). Plants flavonoids have potential for reducing risk of chronic heart disorders. Flavonoids such as quercetin displayed anti-oxidants activities by neutralizing free radicals and preventing oxidative cells and tissues damage (Shatakshi et al. 2025).

In the present study, higher moisture content, protein, crude lipid, crude fiber, carbohydrate and ash contents were observed in the ethanol leaves extract of *E. indica*. Higher moisture content in plants enhances growth and development of spoilage microorganisms and the activities of hydrolytic enzymes (Keyata et al. 2020). Dietary fibre serves important function in human health by enhancing water retention capacity during gastrointestinal transit (Roboul et al. 2017). Lipid serves as major source of high energy metabolites and fat-soluble vitamins that have important roles in biochemical reactions (Abubakar et al. 2025). Crude fibre facilitates the production of bulkier and softer stools (Roboul et al. 2017). Dietary fiber improves the digestion of foods, enhances the absorption of foods in the large intestine, stimulates peristalsis, and prevents constipation (Ogungbenle and Omosola 2015). High-fiber diets have potential to reduce risk of many chronic diseases including diabetes, obesity, coronary heart disease, hypertension, and colon cancer (Ikewuchi and Ikewuchi 2008, Tadessea et al. 2025). Proteins enhance tissues development and promote replacement of damaged tissues in the body (Tadessea et al. 2025). Proteins consist of amino acids, which are required for building and maintaining body tissues (Olufunso et al. 2019). Ash content of food substances is an indicator of minerals content of plants based foods (Abubakar et al. 2025).

The current finding indicated that ethanol leaves extract of *Eluesine indica* contain significant amount of sodium, potassium, magnesium, calcium, zinc, copper, and iron. The K^+/Na^+ ratio is a vital index for high blood pressure and coronary heart diseases (Morrissey et al. 2020). Magnesium serves important function in protein metabolism, regulation of cardiovascular system, energy release from muscle, bones formation, and regulation of body temperature (Allen and Sharma 2019, Gragossian and Friede 2019, Akram et al. 2020). Copper plays a vital role in formation of red blood cell and absorption of iron in gastrointestinal tract (Akram et al. 2020). Calcium is a vital agent for blood clotting, bone and teeth development (Abubakar et al. 2022, Aliyu et al. 2025). Other functions of calcium in the body include regulation of vasodilatation and vascular contraction, impulse transmission, muscle contraction, intracellular

signaling, and hormones synthesis (Catharine et al. 2018). Zinc serves many biological functions including cell growth and development, sexual maturity, fertility, and tissues formation (Baltaci et al. 2018, Akram et al. 2020). It also serves significant function in pain relief, proliferation and maturation of immune cells, hair development, regulation of oxidative stress, activation of signal transduction, and gene expression (Kimura and Kambe 2016, Baltaci et al. 2018, Aliyu et al. 2025).Copper plays a vital role in bone formation, hematopoiesis and serves as cofactor of enzymes particularly catalase, ferro-oxidase, tyrosinase and cytochrome oxidase (Abubakar et al. 2025). Iron is an essential component of haemoglobin, a pigment responsible for transportation of oxygen in the blood (Akram et al. 2020). It regulates the activities of many enzymes and proteins involve in oxidation reduction reactions, hemoglobin synthesis, oxygen transport, cellular growth and development (Yiannikourides and Latunde-Dada 2019, Akram et al. 2020).

In this finding, a reasonable amount of phytates, oxalate, tannins, and saponins was observed in the ethanol leaves extract of *Eluesine indica*. Anti-nutritional factors decrease the absorption, digestion, and utilization of nutrients in foods and their products (Aliyu et al. 2025). Phytates bind essential minerals to form insoluble complexes that decrease the bioavailability of minerals by inhibiting their absorption in the digestive tract (Chen and Xu 2023). Phytic acid inhibits the activities of digestive enzymes resulting to decrease in nutrients absorption (Smeriglio et al. 2017). Oxalate inhibits the absorption of Ca^{+} leading to limited amount of calcium for different roles in the body (Unuofin et al. 2017). It binds to plasma Ca^{+} to yield calcium-oxalate complexes that accumulate to kidney stones (Unuofin et al. 2017, Joye 2019). Oxalates bind minerals to form oxalate salts causing serious adverse health effects (López-Moreno et al. 2022).

Tannin combines with protein to form protein-tannin complexes responsible for inhibition of activities of certain digestive enzymes resulting to proteins deficiency (Joye 2019). Tannins hinder the absorption and digestion of nutrients (Yegrem et al. 2021). They bind to proteins, carbohydrates, and minerals to form complexes that preventing their digestion and absorption (Filho et al. 2017). Tannins inhibit the activities of digestive enzymes leading to decrease in digestion of nutrients (Rani and Kumar 2020). Saponin inhibits the digestion of proteins and absorption of minerals in the gut resulting to leaky gut formation (Barky et al. 2017). Saponins inhibit the absorption of glucose and volatile fatty acids (Yegrem et al. 2021). Over stimulation of immune response by saponins causes autoimmune reactions or enhances inflammatory conditions (Sharma et al. 2023). Saponins have been reported to bind minerals to form insoluble complexes which reduce their bioavailability (Sandeep and Ghosh 2020). Prolong binding of saponins with minerals can leads to anemic condition (Abdelrahman and Jogaiah 2020). Saponins inhibit the activities of proteolytic enzymes such as trypsin and chymotrypsin that catalyze the breakdown of dietary proteins into amino acids (da Silva et al. 2021). Alkaloids have been associated with rapid heartbeat, paralysis and in fatal case (Veer et al. 2021). Alkaloids disrupt signal transmission in nervous system (Veer et al. 2021). Alkaloids inhibited the activity of cholinesterase causing certain neurological disorders (Veer et al. 2021).

Conclusions

The ethanol leaves extract of *Eleusine indica* contain significant amount of proximate components and minerals content. Thus, *E. indica* contain essential nutrients required for tissues development and normal health maintenance. However, the plant extract demonstrated significant amount of phytochemicals suggesting its medicinal properties.

Conflict of Interest

No conflict of interest declared by the authors.

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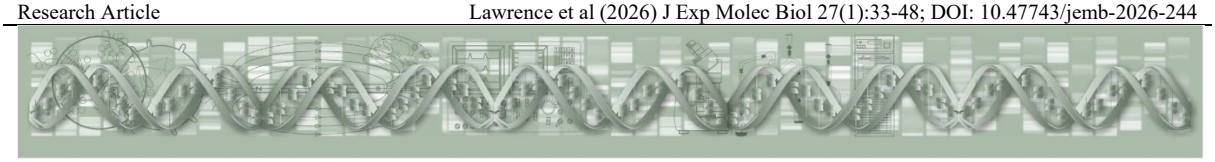

PARADE TOWARDS GREEN REVOLUTION: LARVICIDAL ACTIVITY OF *CATUNAREGAM SPINOSA* AGAINST *AEDES AEGYPTI*

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Abstract

Dengue is a common vector-borne disease prominent in tropical and subtropical climates. It causes losing of millions of human lives per year around the world. *Catunaregam spinosa* is an underrated medicinal plant in Sri Lanka which rumored in possessing larvicidal activity against mosquitoes. Present study investigated the toxicity of seed extract of *C. spinosa* against fourth instar larvae of *Aedes aegypti* L to provide a scientific validation to the embedded property and to support the ethnobotanical vector control approaches. Mosquito larval cultures exposed to a series of concentrations (75.0, 125.0, 250.0, 500.0, 1000.0 mg L^{-1}) showed concentration dependent mortalities and teratogenic effects after 24 hours. Statistical analysis computed 24 h, LC_{50} as 233.67 mg L^{-1} and LC_{90} as 659.93 mg L^{-1} reporting a moderate larvicidal activity. Preliminary phytochemical analysis revealed the presence of alkaloids, coumarins, saponins and flavonoids. Presence of butanoic acid, octadecanoic acid, n-hexadecenoic acid and palmitic acid along with 19 compounds were identified using Gas Chromatography Mass Spectrometry. In conclusion, the study unveils a lodged property in an abandoned plant in Sri Lanka whilst supporting the green-revolution and sustainable health system for future developments of bio-larvicides using natural compounds available in *C. spinosa*.

Keywords: *Aedes aegypti*, bioinsecticide, *Catunaregam spinosa*, green revolution, vector control

Introduction

Mosquito diseases spreading via parasites, bacteria or viruses account for more than 700,000 worldwide annual deaths. Malaria (parasite) and dengue (virus) transmitted by *Anopheles* and *Aedes* mosquitoes are responsible for more than 3.4 million confirmed cases and 40,000 deaths in every year [1]. Sri Lanka as a tropical country provides preferable conditions for rapid life cycles completion of *Aedes aegypti* L. Number of cases of dengue has consistently increased in Sri Lanka since start of 2023 which is three times greater than that of corresponding period in 2021 and 2022 [2].

Life Cycle of *Ae. aegypti*

Chikungunya, zika virus, yellow fever, Japanese encephalitis, dengue and dengue hemorrhagic fever are transmitted by mosquitoes belongs to genus *Aedes. Aedes aegypti* mosquito life cycle is consisted of four developmental stages consisting eggs, larvae, pupae and adults (Figure 1).

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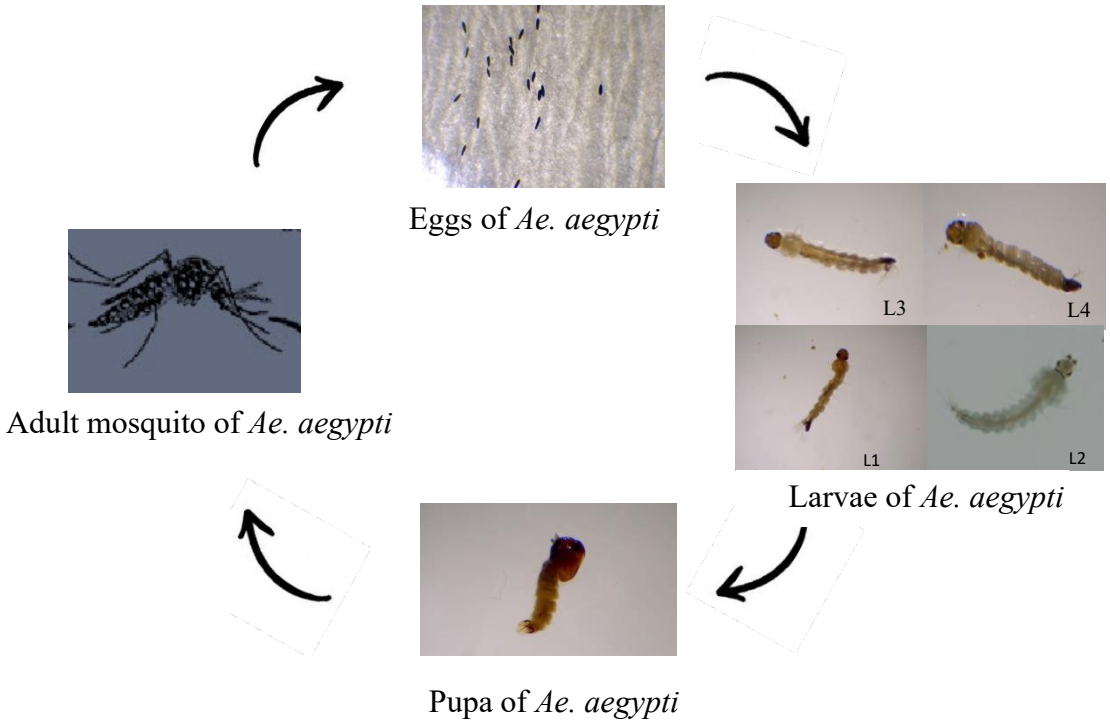

Figure 1. Development life cycle of *Ae. aegypti*

Mosquitoes of *Ae. aegypti* lay about 200 eggs per oviposition and only takes 8 days to complete the life cycle. First two larval stages are formed after first two days of egg hatching and the third instar remains in the next two days. Fourth instar can be observed on the fifth day and on the sixth and seventh days, fourth instar develops into a pupa as an immature adult. Later the mature adults can be seen on the eighth day of mosquito life cycle. *Ae. aegypti* eggs are resistant to dryness which can even last for a year without losing the viability and yet get hatched and produce larvae soon after contacted with water.

Vector Control Approaches

Over the past decades mosquito vector control approaches were linked mostly with chemical, and physical attributes where the current traits are articulated with molecular and ethnobotanical approaches.

Chemical, physical and molecular approaches

There are several principal interventions in vector control approaches where the mosquito management is primitively composed of eliminating sources and the respective vectors of spreading the disease. The approaches are attributed with chemical, physical, molecular and ethnobotanical perspectives. Chemical perspectives such as insecticides, larvicides and non-chemical perspectives including mass trapping of larvae, oil coating, use of larvivores fish, habitat management, suppressing the population via genetically engineered techniques involve in vector control approaches.

Use of mosquito repellents, insecticide treated mosquito nets, covering arms and legs with long clothes, using nets to screen mosquitos at the open doors and windows are common physical preventive measurements in the current society of Sri Lanka.

Deficiency and the absence of vaccines trigger the inauguration of new vector control strategies via investigating different biological aspects to combat the surging mosquito population. Studies have been conducted for reducing the mosquito population by employing genetically engineered male mosquitos where the introduced fatal genes are transmitted to offsprings causing them to die before getting sexually matured. For an instance, Molecular Medicine Unit

of Faculty of Medicine, University of Kelaniya, Sri Lanka conducted a mass project of releasing 100,000 of sterile male mosquitos which made sterile by Sterile Insect Technique (SIT) to a dengue abundant area with the aim of reducing offsprings by suppressing the mating and oviposition frequency of adults [3]. It revealed remarkable reduction of mosquito population at the end of the study period.

Further researches mentioned the use of dispensers that emit neurotransmitters such as neuropeptide Y less attracts the mosquitos to humans. Developing nano technology integrated insecticides are an evolving concept where fungi, bacteria, algae or plant extracts are used as carriers to reduce and stabilize the preferred compounds into nanoparticles [4]. Balaraman et al. (2022) used aqueous extracts of *Sargassum myriocystum* to synthesize Titanium Dioxide nanoparticles (TiO_2-NPs) against *Ae. aegypti* and *Culex quinquefasciatus* [5]. Manojkumar et al. (2023) estimated the LC_{50} and LC_{90} of Zinc Oxide nanoparticles (ZnO-NPs) developed using *Brassica oleracea* var. botrytis as 76.03, 190.03 ppm which tested against the fourth instar of *C. quinquefasciatus* mosquito larvae [6].

Ethnobotanical approaches

High cost, advanced technologies, harmful environmental impacts and resistance build up from insects decelerate the demand for chemical insecticides where additional strict rules and regulations upon synthetic insecticides arouse the interest on botanical insect management strategies. Application of ethnobotanicals is a raising technique in controlling mosquitoes as of the easy degradability, less persistence in the environment, less harmfulness to vulnerable parties and the cost effectiveness. Bio-insecticides are usually derived from bio active compounds extracted from plant materials or microorganisms [7]. Entamopathegenic fungi orders such as Entamophthorales, Hypocreales and predatory fish genera such as *Gambusia*, *Poecilia* and copepods are used in managing mosquito populations [8,9].

Diverse phytochemical compositions found over 400,000 plant species unlocks the pathways in developing eco - friendly insecticides. Leaves, fruits, root, stem bark and seeds belong to different plant families such as Fabaceae, Piperaceae, Asteraceae, Apocynaceae and Euphorbaceae possess significant toxicity against *Ae. aegypti* [10-13]. Kumar et al. (2012) studied toxicity of 15 local plants grown in New Delhi against larvae of *Ae. aegypti* and revealed 10 species as potent sources where the *Achyranthes aspera*, *Zingiber officinalis*, *Ricinus communis*, *Trachyspermum ammi* and *Cassia occidentalis* exhibited significant activity as the Median Lethal Concentration (LC_{50}) ranged from 55.0 to 74.67 mg L^{-1} [14]. Organophosphates, carbomates and pyrethroids are historically used synthetic larvicides and essential oils, terpenoids, phenyl propanoids, thiophenes, alkaloids, tannins, coumarins, geraniol are naturally occurring mosquitocidal compounds [15]. Plants belong to family Rubiaceae show larvicidal activity against *Ae. aegypti* at different concentrations. Methanolic and acetone leaf extracts of *Gardenia ternifolia* found in Kenya exhibited 32.01 mg L^{-1} and 83.31 mg L^{-1} of LC_{50} respectively [16]. Carvalho et al. (2014) studied larvicidal activity of *Spermacoce latifolia* found in Brazil and revealed LC_{50} of acetone, methanol and n-Hexane extracts as 574.0, 625.0 and 415.0 mg L^{-1} respectively [17].

Catunaregam spinosa

Catunaregam spinosa is an underrated medicinal plant in Sri Lanka which belongs to family Rubiaceae. *C. spinosa* grows up to 1-10 m as a large deciduous thorny shrub or small tree (Figure 2a). Its shiny leaves are arranged in groups of three and are 0.75-3.5 inches in length and 0.4-1.2 inches in width (Figure 2b). White, pale yellow flowers of *C. spinosa* emit a honey-like fragrance and bloom at the terminals of the lateral branches, either singly or in clusters (Figure 2c). The berry-like fruits are small, measuring 2-6 inches, with a hard pericarp and fleshy interior (Figure 2d). Seeds are smooth, compressed, oval shaped, and embedded in a dark, fetid pulp with a prismatic shape (Figure 2e).

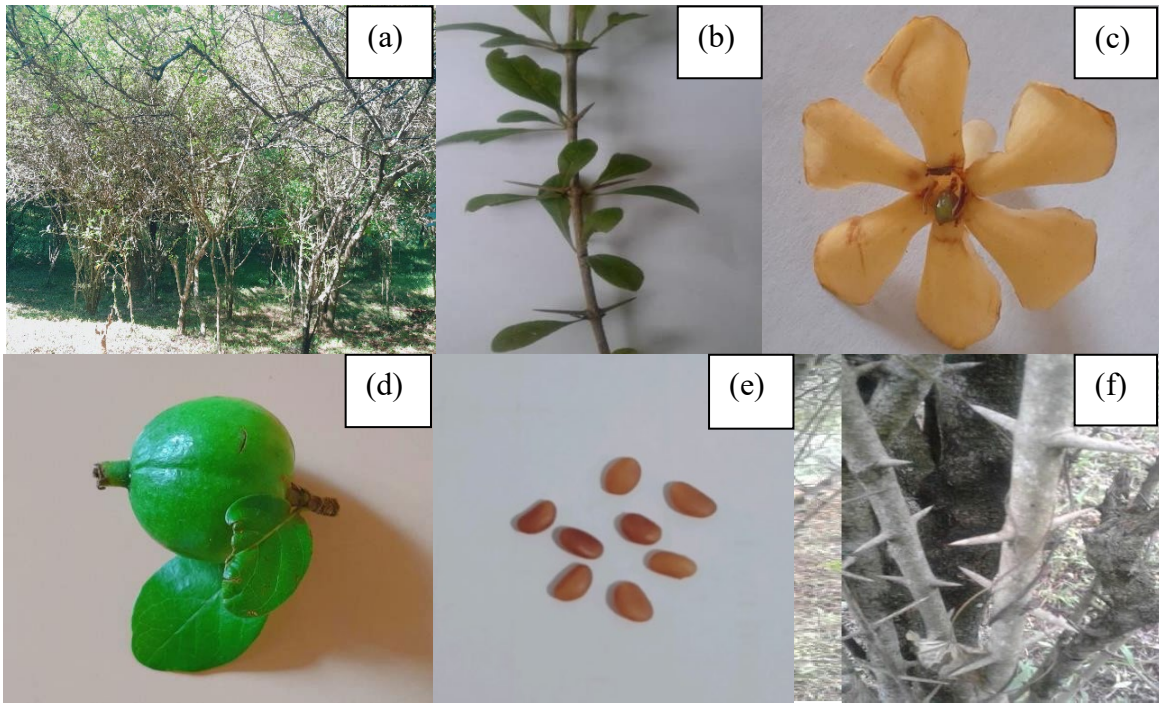

Figure 2. (a) habitat, (b) leaves, (c) flower, (d) fruit, (e) seeds, (f) spines of *C. spinosa*

The plant possesses crucial pharmacological properties of anti-inflammatory, anti-oxidant, cytotoxicity, anti-bacterial, insecticidal, piscicidal, anti-microbial and anthelmintic in different parts including leaves, fruits, stem bark, roots and seeds [18-22]. Glycosides, triterpenoids, saponins, flavonoids and alkaloids are major secondary metabolites found in *C. spinosa* which are responsible for mentioned crucial properties [23,24].

Global and Sri Lankan Distribution

C. spinosa is distributed among several tropical and sub-tropical countries (Bangladesh, Sri Lanka, Indonesia, China, Malaysia, Vietnam, most of the jungles in India and in African countries such as Tanzania, states of Texas, California and Arizona in United States, Middle East countries) [25] where the same regions are high prevalence of dengue. According to the "Flora of Ceylon", *C. spinosa* employs either as a single plant or small to large clusters in most of the districts in Sri Lanka; Jaffna and Mannar – northern region, Puttalam, Polonnaruwa and Anuradhapura – north central region, Trincomalee and Batticaloa – north eastern region, Matale, Kandy, Badulla, Haldumulla and Rathnapura – central region, Hambantota and Monaragala – south eastern region, Nawinna – south western region [26].

Investigating the larvicidal activity of abundant *C. spinosa* is worth and timely as insecticidal, larvicidal and mosquito repellent activities are less concerned and poorly studied both in worldwide and nationwide context [27-29]. Aboriginal people lived in different localities of the world used *C. spinosa* to repel mosquitoes. Pawar et al. (2008) mentioned applying extracts of fruits on skin by people who lived in Jalgoan District in India [30]. Navinkumar et al. (2019) mentioned employing crushed parts of the plant in paddy fields to repel insects using the strong smell disliked by insects with no proven laboratory studies [31]. Wuillda et al. (2019) reviewed larvicidal activity of secondary metabolites found in 85 plant species against *Ae. aegypti* from the data collected from literatures during 2013 to 2018 where no data was found occupying of *C. spinosa* [32]. Anoopkumar et al. (2020) studied larvicidal activity of petroleum ether, ethanol, acetone and aqueous seeds extracts of *C. spinosa* grown in India against *Ae. aegypti* as the only laboratory experiment over the desired activity [33]. Geological and ecological variations such as rainfall, soil type and temperature play key roles in determining phyto- potencies of plants as of leading different phytochemical profiles and strengths. The variations cause for different

lethal concentrations resulting available for unique regional vector control approaches. Therefore, the present study will provide details about larvicidal activity of *C. spinosa* grown in Sri Lanka which will facilitate in extracting the potential of this under-utilized plant and mitigating the dengue virus spreading, co-currently supporting the global trend of green revolution. Also, the titled study used larvae as the test organism to improve the efficacy of using the property before mosquitoes completing their life cycle.

Materials and Methods

Larvicidal activity of *C. spinosa* grown in Sri Lanka was investigated establishing suitable mosquito cultures, collecting authenticated plant materials, extracting and analyzing the phytochemicals and conducting the assay according to the guidelines by WHO.

Establishment of mosquito culture

Initial *Ae. aegypti* mosquito colony was established using eggs collected from wild female mosquitoes caught from Medical Officer of Health area, Ragama, Sri Lanka and maintained in the insectary of Molecular Medicine Unit, Faculty of Medicine, University of Kelaniya at 26 ºC and 75± 5 % relative humidity under 12:12 (Light: Dark) photoperiod [34]. Mosquito rearing and colony maintenance were followed according to the guidelines by Standard Operating Procedures (SOP) on rearing and maintenance of *Aedes* mosquito colonies in the insectary by National Research Council, Sri Lanka. The collected eggs were hatched in hatching trays contained 2.0 L of conditioned water. Larvae were fed with diet containing 50 % (w/v) tuna meal (12.5 g), 36 % (w/v) bovine liver powder (9.0 g) and 14 % (w/v) brewer's yeast (3.5 g) dissolved in 100.0 mL distilled water. Approximately 1.5 mL of diet was randomly added into the larval tray. Pupae were collected into bowls after 5 days of eggs hatching using pasture pipettes. Bowls of pupae were placed inside plastic cages (24 x 24 x 24 cm) for adult emergence. Adults were fed with 10 % sucrose solution prepared by dissolving 50.0 g of glucose and 5.0 mL of B - vitamin in 500.0 mL distilled water soaked in cottons. Adult females deprived of sucrose were fed with cattle blood. The five hundred of fourth instar larvae were collected as the test samples from the second generation of the same adults reared under the similar conditions and each concentration was tested for sixty larvae.

Collection of plant materials

Seeds were obtained from five months old matured fruits of *C. spinosa* grown in Ayurveda Herbal Garden, Haldumulla, Sri Lanka (6º45′42″ N 80º53′59″ E) and the plant was authenticated from National Herbarium, Department of National Botanic Gardens, Peradeniya (ID-43). Collected seeds were air dried for three days and pulverized using an electric grinder to obtain powdered seeds.

Preparation of seed extract

The stock solution of seed extract of *C. spinosa* (SECS) (1000.0 mg L^{-1}) was prepared dissolving 1.0 g of powdered seeds in 1.0 % (v/v) Dimethyl Sulfoxide (DMSO) (≥ 99.5% Sigma-Aldrich, St. Louis, M063178, USA). One percent (v/v) DMSO was prepared mixing 10.0 mL of DMSO and 990.0 mL of water conditioned for one week. The stock solution of SECS was kept overnight for effective extraction of phyto - constituents and filtered through a muslin cloth. The stock solution was diluted to prepare concentrations of 75.0, 125.0, 250.0, 500.0, 1000.0 mg L^{-1} and tested for mortality of *Ae. aegypti* fourth instar larvae.

Larvicidal assay

The assay was conducted according to the guidelines by WHO (2005) [35]. Twenty larvae were released into each container with 200.0 mL of test solutions. Each concentration was replicated at 26 ˚C. Pre-conditioned water was used as the negative control and 1.0 % DMSO was the solvent control which were also triplicated. Both negative and solvent controls were added with twenty larvae in each. Larvae were not fed during the test period to avoid possible interventions

that can be caused on the mortality. Number of dead and moribund larvae were counted after 24 h to calculate the mortality.

Statistical analysis

Regression analysis was employed using logit model to compute $LC_{50,}$ Maximum lethal concentration (LC_{90}), 95% fiducial limits of the upper confidence limit (UCL) and lower confidence limit (LCL) using SPSS (Statistical Package of Social Sciences) software version 24.0. A factorial analysis of variance (ANOVA) was performed using concentration as the variable to find the significant difference between that parameter on larval mortality. Larval mortality was served as dependent variable and concentration was treated as the fixed factor.

Analysis of bioactive compounds

Bioactive compounds of SECS were analyzed using preliminary chemical analysis and advanced method of Gas Chromatography Mass Spectrometry (GC-MS).

Preliminary chemical analysis

Phytochemicals contained in twenty grams of powdered seed sample were extracted in to a mixture of 2.0 mL of DMSO and 198.0 mL of pre - conditioned water in a water bath at 45 ˚C for 4 h. The extract was subjected to preliminary phytochemical analysis where the color intensities, color changes and precipitate formations were recorded.

GC-MS analysis

The active chromatographic fraction of the seed extract was further analyzed for phyto - compounds by GC-MS (Model – 7890B, Agilent Technologies, Santa Clara, California, USA). The methodology used by Mdoe et al. (2014) was occupied as the reference protocol with modifications for phytochemical analysis [36]. Column (length – 30 m, diameter – 250 µm, thickness - 0.25 µm) was consisted of HP-5MS 5% phenyl Methyl Siloxane and helium was the carrier gas at flow rate of 1.0 mL/min. Detected compounds were identified and relative percentages were clarified using National Institute of Standards and Technology (NIST) library as the external standard database. Retention time and area percentage of each component were evaluated.

Results and discussion

The results exhibited positive correlation between SECS and larvicidal activity with the presence of crucial active compounds.

Larvicidal activity

The SECS exhibited significant larvicidal activity against fourth instar larvae of *Ae. aegypti* within 24 h. The larvae mortality was observed following several structural and behavioral changes. Major structural damages were observed in the head, abdomen and thorax of larvae (Figure 3a).

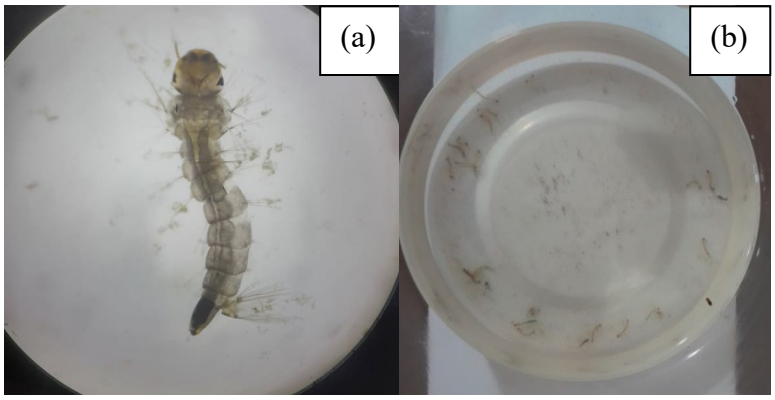

Figure 3. (a) Structurally damaged larvae of *Ae. aegypti*; (b) Dead bodies of larvae floated on the surface and settle down at the bottom of the test container

The behavioral changes and the mortality durations depended on the test concentrations. At the beginning of introduction of larvae showed no distinct behavioral changes in any of the concentrations yet nearly after 10 min they restlessly swam between top and the bottom of the solution body, notably in the highest concentration of 1,000.0 mg L^{-1}. After 20 min, restless swimming and the rapid movements were slowed down and the dead bodies started floating on the surface and some settled at the bottom of the container (Figure 3b). Larvae treated in low concentrations showed normal swimming behavior soon after the introduction and attained moribund stage as of increasing the exposure time.

The SECS recorded concentration dependent significant mortality (p = 0.003) with LC_{50} of 233.67 mg L^{-1} (UCL; 282.52 mg L^{-1} – LCL; 184.80 mg L^{-1}) and LC_{90} of 659.93 mg L^{-1} (UCL; 708.85 mg L^{-1} - LCL; 611.07 mg L^{-1}) against fourth instar larvae of *Ae. aegypti* (Figure 4). Mean mortality percentage (MMP) was < 20 % up to the concentration of 125.0 mg L^{-1} and a steep up to 66.66 % starting from 250.0 mg L^{-1} (Table 1). At the highest concentration of 1,000.0 mg L^{-1} all twenty larvae were died within 24 h. Among the scarcely found literatures, the most recent study revealed the larvicidal activity of petroleum ether, ethanol, acetone and water extracts of SECS grown in India against laboratory reared *Ae. aegypti* which reported LC_{50} as 184.257, 465.224, 248.680 and 210.212 mg L^{-1} respectively [33]. They revealed LC_{90} of aqueous seed extract as 3205.89 mg L^{-1}. The recorded LC_{50} of hydroalcoholic seed extract of Sri Lanka (233.67 mg L^{-1}) is slightly higher that of recorded by Anoopkumar et al. (2020) and conversely, the LC_{90} of Sri Lankan variety (659.93 mg L^{-1}) is significantly low; more than quarter of the recorded LC_{90} by Indian variety. Based on the results, Sri Lankan variety possesses moderate larvicidal activity which is preferable in developing efficient bio-larvicides as well, as only a lesser concentration is required for the mortality of 90 % of *Ae. aegypti* larvae population. Most of the studies have used solely the chemical solvents in extracting phyto-constituents wherein the present study only used pre-conditioned water along with DMSO <2.0 % (v/v). Roots and stem barks are highly potent sources of secondary metabolites as of most studies have been focused on and reported significant toxic values. Results of our study will provide information to the field where knowledge was lack on SECP.

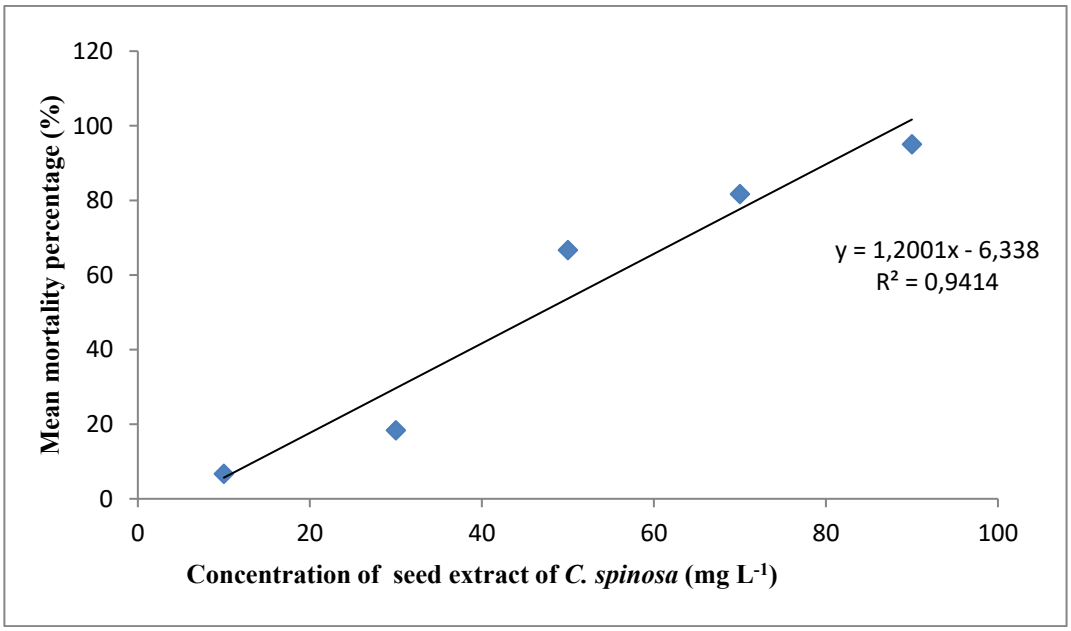

Figure 4. Graphical representation of concentration dependent larvicidal activity of SECS against fourth instar larvae of *Ae. aegypti* after 24 h

Table 1. Concentration dependent larvicidal activity of SECS against fourth instar larvae of *Ae. aegypti* after 24

Concentration (mg L^{-1})	MMP ± SD
75.0	6.66 ± 0.88
125.0	18.33 ± 0.88
250.0	66.66 ± 0.88
500.0	81.66 ± 0.88
1000.0	95.00 ± 2.00

Sub - acute toxicity was observed at low concentrations of the extract as the number of larvae developed into pupae was lesser compared to that of in the controls. Sub - acute toxicity can reduce the fertility, fecundity and life span of developed larvae into adults after the few days of their survival. No mortality was observed in the controls instead normal larvae and pupae developments along with the adult emergence after 8 - 9 days of eggs hatching.

Preliminary analysis

The SECS showed positive results for alkaloids, saponins, tannins, flavonoids, coumarins and triterpenes. Insolubility of alkaloids, flavonoids, coumarins, fatty acids and triterpenes in water was overcome by dissolving the powdered seeds in DMSO < 2.0 % (v/v).

GC-MS analysis

Present study identified 19 chemical compounds in SECS using GC-MS analysis after comparing with known compounds in NIST database (Table 2). The chromatogram is presented in Figure 5. Butanoic acid, palmitic acid, n-hexadecanoic acid, octadecanoic acid were the major compounds identified which could be responsible for the larvicidal activity of SECS.

Table 2. Phytochemicals detected in SECS using GC-MS analysis

No.	Retention time (min.)	Compound name
1	2.986	Glycerin
2	3.439	3-methylbutanoic acid/ Isovaleric acid
3	4.955	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one
4	6.731	Unknown
5	6.790	Unknown
6	7.982	Pyranone
7	8.351	Unknown
8	9.608	2-(ethoxymethyl) oxirane
9	11.897	Tetradecanoic acid
10	12.610	2-Methoxy-4-propylphenol/ Cerulignol
11	13.148	8-methyltetrahydro-4H-(1,3)-dioxino(5,4-d)(1,3) dioxepin-9-ol
12	16.153	2-Propanone, 1-hydroxy-3-(4-hydroxy-3-methoxyphenyl)-
13	17.016	Neophytadiene
14	17.988	Palmitic acid, methyl ester
15	18.387	Hexadecanoic acid, methyl ester
16	19.306	n-Hexadecanoic acid
17	19.897	Phytol
18	20.235	Octadecanoic acid/ oleic acid
19	21.474	Triethylene glycol monododecyl ether

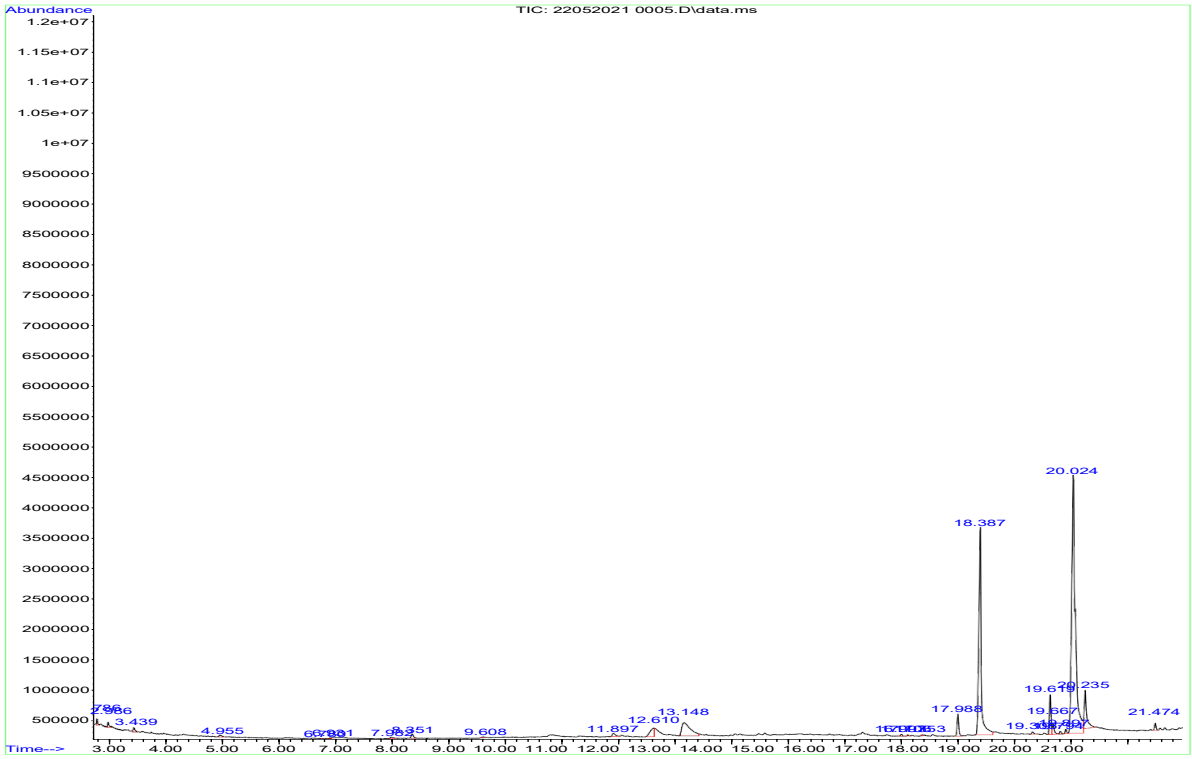

Figure 5. GC-MS chromatogram of phytochemicals detected in SECS using GC-MS analysis

Phytochemical constituents and plausible mechanisms

Generally, classes of secondary metabolites such as alkaloids, saponins, tannins, flavonoids, coumarins, triterpenes, acetogenins, lignans, fatty acids and naphthoquinones stand for larvicidal/ insecticidal activity of the plants [37-41]. Most of these compounds were identified in the SECS grown in Sri Lanka using preliminary and GC-MS analysis. The same compounds were reported for major physiological malformations in the body and to inhibit the nerve signal transmission in the Central Nervous System (CNS) of larvae. Saponin causes stomach toxicity via physiological and morphological damages to midgut epithelial cells of mosquitoes where the excessive absorption causes cells to get vacuolated and vesicles to release energy for detoxification, leading cells to die [42]. Saponin as one of the components might be caused the physical damages observed in the test larvae. Also, saponins lead the retarded development, decreased fecundity and the increased mortality of larvae even after their survival where the similar traits were observed in our test samples. Damaged and disrupted abdomen of larvae and attaining them moribund stage would be caused by the presence of tannins as those are reported in attacking the integrity of digestive track. Because tannins decrease the nutrients intake via hardening the cell membranes and cause malformations due to the excessive ingestion and fumigation of toxicants [43]. Flavonoids as a substance found in the extract might strongly inhibited the Acetyl Cholinesterase (ACh) activity in larvae causing them to paralyze and also to affect their detoxification system by interrupting the activities of Glutathione S - Transferase (GST) and esterase [44]. The detected fatty acids also undergo the same mechanism of inhibiting ACh in CNS [45, 46]. Both tannins and flavonoids are not only neurotoxicants but generate the free radicals to damage the DNA, where the respective formations cause the malformations in proteins resulting mosquitoes to die [33]. As of SECS is reported with significant level of free radicals that can be caused DNA damages of larvae [33]. The synergetic effect of neurotoxicity and the free radical generation can be one of the reasons for death of *Ae. aegypti* larvae. Also, the identified compound composition with several pharmacological activities including anti-microbial activity might enhanced the productivity of the extract as it diminishes the host-parasite interaction in the mosquito midgut. However, a combined chemical composition with multiple properties is more effective than using a single or few compounds with limited activities for mosquito management strategies which is well-employed in the SECS extract.

In the current local market, there is already a bacterium-based product (*Saccharopolyspora spinose*) but not an ethnobotanically developed formulations where the produced knowledge in the present article will raise the competition of the natural ingredients-based products to discourage the use of synthetic mosquitocides, and finally to make mosquito control in Sri Lanka hassle-free and effective. Also, the unveiled information will allow to get the maximum value of a well-grown yet underrated medicinal plant in Sri Lanka.

Impact on non-target organisms

Field trials are to be performed on the non-target organisms and none of the literatures mentioned the side effects of SECS over unbalancing of the ecosystem or undesirable effects on non-target organisms and human health. Supporting the use of less toxic SECS, a highly toxic chloroform seed extract of *Annona mucosa* which contains *squamosin* as the larvicidal compound (LC_{50}: 0.01 mg L^{-1}) showed no toxic effect on two predators of *Ae. aegypti* - *C. bigoti* and *Toxorhynchites theobaldi* and also to the mammals based on the results against human leukocytes [47].

Conclusions

Mortality of *Ae. aegypti* larvae exposed to different concentrations of SECS was concentration dependent and significant which recorded LC_{50} of 233.67 mg L^{-1} and LC_{90} of 659.93 mg L^{-1} possibly due to the presence of saponins, alkaloids, coumarins, tannins, flavonoids and fatty acids. All these results explicit the naturally occurring larvicidal potential of an underrated

medicinal plant of *C. spinosa* to be successfully use as a safe alternative for chemical larvicides to combat the dengue mosquitoes and for a sustainable health system of Sri Lanka amidst of promoting nature-based products.

Future direction

Indiscriminate use of chemical adulticides and larvicides has become a massive disaster in terms of both environmental and human sustainability. Unfortunately, chemical mosquitocides are favored by users due to user-friendly approach, effectiveness and long-lasting activity which is affirmed by different product modifications. Unlike chemical products, if a bio-larvicide is developed using SECS will be easily degradable, less harmful to both users and non-target species and will be low probability in developing resistance from target species. Even though, the larvicides derived from natural resources are safer than the chemical products, it is concerning as the conventional use of whole plant would affect the diversity and the conservation rationale of the plant as mass usage of raw materials. Anyhow, unique and optimized protocols for extracting target compounds from *C. spinosa* will allow to use only small quantities. Also, usage of suitable adjuvants as buffering and antimicrobial agents, UV protectant, emulsifiers, stabilizers and for adhesion will ensure the firmness and durability of *C. spinosa* based product facilitating to develop cost-effective and eco-friendly bio-larvicides. As the reported LC_{90} of SECS was noticeably low, a competitive and commercially worth bio-larvicide can be developed. Further investigations on optimum concentration and applying frequencies will improve the product efficacy and the survival in different environmental conditions. Further, the future direction of using *C. spinosa* can be oriented for field trials in studying persistency of the formulation and its effectiveness, effects of non-target species such as important worms, snails and small insects etc. Studying the effects on soil conditions such as soil pH and sterility will be important in mitigate the side effects of the product.

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Authors' contribution
PK Lawrence: Conceptualization, Writing - Original Draft, Review & Editing. WTPSK Senarath: Writing - Review & Editing, Supervision. M.D. Hapugoda: Writing - Review & Editing, Supervision.

Statements and declarations

Ethical considerations
Not applicable.

Declaration of conflicting interest
Authors have declared that no competing interests exist.

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Data availability
No data was used for the research described in the article.

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