



mRNA Expression of S1PR-1 in DMBA Induced Leukemia in Wistar Rats Treated with Ethanol Extract of Hydrocotyle Verticillata and Laportea Aestuans Leaves

Odo, Christopher Emeka ^{a*}, Uwakwe Augustine A. ^a
and Ogunka-Nnoka Charity ^a

^a Department of Biochemistry, Faculty of Science University of Port Harcourt, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. All Authors were involved in the design of the study while author OCE managed the experimental analysis of the work, statistical analysis, wrote the draft of the manuscript as well as searching for the literatures. Authors O-NC and UAA corrected the manuscript, made inputs. All authors finally read and approved the final manuscript.

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ABSTRACT

Aim: Leukemia has been linked to mRNA expression of Sphingosine-1-phosphate receptor-1(S1PR-1) and considering multiple reports on the roles of phytochemicals in Hydrocotyle verticillata and Laportea aestuans, this study aims to evaluate the effects of administration of ethanol extracts of the 2 plants in regulation of mRNA expression of S1PR-1 in leukemia induced by 7,12-dimethylbenz(α) anthracene (DMBA) in rats.

Methods: Leukemia was induced by a series of subcutaneous injection of 27% of DMBA except for the normal and DMSO groups at interval of 4 days for two weeks. The ethanol extracts of the 2 plants with a standard drug was administered for 2 weeks and blood specimens collected by cardiac puncture under anesthesia with chloroform, from the respective groups and into an EDTA container after induction and extract treatments and Lymphocytes was then extracted. Total RNA was extracted and reverse transcribed. The cDNA was amplified on My IQ single color RTPCR yielding threshold cycles for the target and the house keeping genes which was used for mRNA expression fold of S1PR-1.

*Corresponding author: E-mail: christopher_odo@uniport.edu.ng;

Results: The result shows that S1PR-1 was downregulated in the leukemia induced groups 3 to 9 of rats when compared to group 1 and 2. Conversely, after treatments the downregulated gene was found to be upregulated when given the extracts of *H. verticillata*, *L. aestuans* and the standard anti-leukemic drug treated groups. This upregulation was high with the standard drug, then the combined dose of 400mg of both extracts, the 400mg of *L. aestuans*, 400mg of *H. verticillata*, while 200mg of each of the extract of *H. verticillata* and *L. aestuans* was upregulated in the same manner.

Conclusion: This outcome of the present study has shown that the plant extracts upregulated the mRNA expression of S1PR1 in a leukemic rat hence can be used as an adjunct in leukemia treatment.

Keywords: S1PR-1; leukemia; gene expression; phytochemicals; DMBA.

1. INTRODUCTION

Leukemia which is a proliferation of hematopoietic stem cells in the bone marrow is a common malignancy in children and adults that occurs when changes in normal cell regulatory processes leads to an unchecked proliferation of hematopoietic stem cells in the bone marrow [1]. It is a form of metastatic growth which targets the blood; and contains different kinds of blood cells. The normal life cycle of these cellular elements is dictated in part by the bone marrow. In many fora, if the control over white blood cell's life cycle is disturbed, leukemia results. Leukemia rarely take place in rats but radiation and chemicals particularly the polycyclic aromatic hydrocarbons such as 3-methylcholanthrene, 7,12-dimethylbenz(α)anthracene (DMBA), and 7,8,12-trimethylbenz (α) anthracene (TMBA) can give rise to the condition. Leukemia due to DMBA is known to result to thymic leukemia, erythroblastic stem cells, acute lymphoblastic, and acute myelogenous leukemia. Rats exposed to DMBA has been shown to results in the formation of cohorts of DMBA-DNA which gives rise to transversion of chromosome (A to T), in codon 61 at the second base of N-ras gene. This mutation which is point mutation in form, has been discovered to arise due to the removal of a purine as a result of DMBA-DNA adduct synthesis, followed by mis-replication across the unrepaired apurinic site. The genomic changes in the Ras gene were thought to activate many signaling pathways leading to cell proliferation involving mRNA expression of S1PR-1 signaling [2,3].

A fast-acting metabolite of the sphingolipid family known as sphingosine-1-phosphate [S1P] signaling have been attributed to be involved in the modulation of a wide array of numerous physiological and biochemical activities at cellular level. They are class of cellular receptors that are coupled to different G-protein subunits

namely G_{α} , G_{β} , G_{γ} , G_s and G_i molecules. These surface receptors have been linked to exert a very germane role in the development, growth, survival and progression of cancer [4]. It has been noted that S1P signaling is a key regulator of T cell exit from both lymphoid and non-lymphoid tissues, as well as T cell retention in tissue microenvironments such as the germinal center [5]. Numerous studies have demonstrated that S1P signaling is closely associated with leukemia progression. Thus far, focusing on S1P signaling as a promising therapeutic target which involves two main research strategies: one is to reduce the levels of S1P itself while the other is to agonize/antagonize S1P receptors. The current management of leukemia which involves employing radiotherapy and chemotherapy can results in relative amelioration of the symptoms of the ailment and subsequently offers the opportunity for an extended treatment regimen which can results to a relative well-being to the victims of leukemia. The use of whole blood transfusions and antibiotics, as the indications arise, is important if the best result is to be achieved [6].

Existing treatment protocols (chemotherapy, radiotherapy and bone marrow transplant) for leukemia therapy may be quite effective but they pose serious adverse effects on the individual as well as cost of treatment particularly in developing countries therefore, newer means need to be explored which may help to fight the disease and plant derived molecules, or phytochemicals are studied with various disease fighting properties as they exert their action by targeting various signaling molecules that are involved in the process of leukemia. These phytochemicals act preferentially as they target the leukemic cells only, sparing the normal healthy cells. *Laportea aestuans* and *Hydrocotyle verticillata* have been reported to be used by traditional medicine practitioners to treat blood disorders across our local populace without any

supporting scientific studies to justify their use, toxicity studies, and dose relationship. There is dearth of literature on the anti-leukemic effects of the ethanol extracts of *Laportea aestuans* and *Hydrocotyle verticillata*. *Hydrocotyle verticillata* belongs to the family Apiaceae and consists of about 100 species distributed throughout temperate and tropical region of the world. Recent report has shown that it can be found around the marshy regions of the world such as in Bonny Island, a province in oil and gas rich Rivers state, Nigeria. It has been identified just like others as having numerous biological roles with lesser toxic effects and it cheap to source from the environment. *Laportea aestuans*, the West Indian wood nettle, is an annual herb of the Urticaceae or nettle is composed of 22 species [7]. *Laportea aestuans* (L.) Chew is recorded as an ethnoveterinary species that is used for urinary problems of ruminants in Trinidad and Tobago and also to shorten labor and remove the placenta during childbirth [8,9]. Epidemiological studies indicated that consumption of *L. aestuans* inhibits the damaging activities of free radicals in human body [10].

Considering the numerous reports on the possible beneficial and synergistic potentials of phytochemicals that are found in *Hydrocotyle verticillata* and *Laportea aestuans*, this research set out to verify and further develop a yet relatively cheap and locally available natural therapy for leukemias in male and female Wistar rats experimentally induced with this condition. Based on the knowledge that these plants extracts possess anti-oxidant, anti-inflammatory, cytotoxic effects and their use in traditional practice to treat blood disorders, no studies have investigated their anti-leukemic effects *in vivo*. This research aims to evaluate the roles of ethanol extracts of *Laportea aestuans* and *Hydrocotyle verticillata* in regulation of mRNA expression of Sphingosine-1-phosphate receptor-1 (S1PR-1) in experimentally induced leukemia using 7,12-dimethylbenz(α) anthracene in wistar rats.

2. MATERIALS AND METHODS

2.1 Collection of Plants and identification

The plants, *Hydrocotyle verticillata* and *Laportea aestuans* were collected from the field of Bonny Island, Rivers State Nigeria and was identified as *Hydrocotyle verticillata* and *Laportea aestuans* by a taxonomist, at the science faculty, department

of plants science and environmental biology, University of Port Harcourt.

2.2 Preparation of Crude Extract

Whole plant of *H. verticillata* and *L. aestuans* were collected, sorted and washed to remove dirt, weighed and air dried for about 4 weeks until the weight was constant and then pulverized using mill machine at University of Port Harcourt, Biochemistry department. The pulverized samples (1000g) of each of the two plants were kept in an air tight container for further analysis.

2.3 Extraction of the Crude Plant Extracts

The fine powder each of *Hydrocotyle verticillata* and *Laportea aestuans* weighing about 500g was subjected to successive solvent extraction using absolute ethanol in a volume ratio of 1:4 which is 250g powder dissolved in 1000ml of absolute ethanol, soaked for 72hrs, macerated, filtered using filter paper and concentrated/dried in a rotary evaporator in reduced pressure to obtain the dried extract of both plants. The recovered extracts were 50g of *L. aestuans*, and 38g of *H. verticillata* respectively.

2.4 Procurement of Animals

Healthy male and non-pregnant female Wistar rats weighing between 30-33mg for induction phase between the ages of about 3 weeks were used for the study. The experimental animals were maintained under standard husbandry conditions in the animal house and fed with the prescribed feeds as recommended with due regard to the established physical factors.

2.5 Groupings and Induction of Wining Wistar Rats with 7, 12- dimethylbenz [α] Anthracene

A total of 72 rats were used for the leukemia study and partitioned into 9 groups of 8 rats each. Three weeks (21 days) old wistar rats were grouped and acclimatized for five days. On the sixth day, being their 26th day, all animals weighing between 30-33g were induced with pulse doses of 25% of 7, 12- dimethylbenz [α] anthracene (DMBA) except for the normal control and DMSO group. The induction process was repeated at intervals of four days for duration of two weeks (14 days), establishment of leukemia induction, was carried out before the commencement of the various extract

administrations. Due to the insolubility nature of 7, 12- dimethylbenz [a] anthracene, dimethylsulphoxide (DMSO) was used as vehicle for its conveyance.

At the end of the 14 days durations, blood samples were collected by cardiac puncture under anesthesia with chloroform, and dispensed in a container (EDTA, heparin) for rats of control, DMSO, and selected rats from the other pool of rats and analyzed for mRNA expression of S1PR-1 respectively to establish the induction of leukemia. These were recorded and reported before the various treatment protocols.

2.6 Administration of the Plants Extracts to the Experimental Animals

The administered dose was calculated using the following formula:

$$\text{Dose} = \text{standard dose} \times \text{body weight}/1000\text{g}$$

The stock solution was prepared on a weekly basis and administered 1ml for 200mg extract treated rats and 2ml for 400mg extract treated rats making adjustments as their body weight increases. Since the plant extracts are mainly soluble in water, distilled water was used for the stock preparation.

2.7 Collection of Blood Samples

At the end of the extract administration, blood samples were collected by cardiac puncture under anesthesia with chloroform, from the respective groups and dispensed in a container (EDTA, heparin) and analyzed for expression of S1PR-1 to verify the extent of leukemia treatment. Blood specimens were again collected after the extract administration. These were recorded and reported.

2.7.1 Separation of Lymphocytes using Lymphocyte Separation Medium [LSM]

A rapid separation of lymphocytes from whole blood using centrifugation technique through the Ficoll-sodium metrizoate solution where an equal volume of diluted blood specimen was layered on the LSM reagent and spun at a low centrifugal force for a short duration. This process leads to the sedimentation of granulocytes and red cells to the down of the centrifuge tubes while lymphocytes and platelets settle at the upper interface and collected. Defibrinated or heparinized human blood is diluted with

phosphate buffered saline in a 1:1 proportion; layered over the separation medium, and spun at a low speed for 30 minutes which results in the formation of different cellular layers. The pellet which is formed is comprised mostly of erythrocytes and granulocytes which have migrated through the gradient. The cells were then washed again, with buffered balanced salt solution and resuspended in RNA-LATER solution and stored at temperature of 2-8°C for further downstream applications.

2.7.2 Extraction of RNA from the extracted Lymphocytes (GENEzol™ TriRNA Pure Kit)

The assay principle for the GENEzol™ TriRNA pure kit which rely on Guanidine isothiocyanate and phenol spin column separation platform gives a high throughput Total RNA from a vast array of specimens. In the process, the specimen are homogenized in GENEzol™ Total RNA isolation reagent without using the chloroform or the isopropanol RNA precipitation phase. Following specimen homogenization, simply bind, wash and elute the high-quality Total RNA in RNase-free water and use in a variety of other sensitive downstream applications.

The procedure involves samples homogenization and lysis using Genezol™ TriRNA Pure Kit, followed by RNA Binding, RNA washing, and RNA elution to elute the purified RNA. The concentration and purity of the extracted RNA was determined using Nano drop spectrophotometer (ND-1000). This was read, recorded and stored at -2°C for further downstream analysis. The extracted RNA is now a template RNA which was used for cDNA synthesis.

2.7.3 Synthesis of cDNA from the extracted Total RNA (FIREScript Reverse Transcriptase)

The extracted RNA was converted to cDNA using the FIREScript RT cDNA Synthesis KIT. The process involved using 1µl of Reverse Transcriptase, 2µl of 10x reaction buffer, 0.5µl RNase Inhibitor (Ribogrip), 0.5µl of primers with a 5-µM concentration and 10µl of the RNA sample (at 50ng/µl). Nuclease Free Water was used to balance the reaction volume to 20µl. The thermocycling conditions were as follows; annealing at 25°C for 10minutes, Reverse Transcription at 45°C for 30 minutes and Enzyme inactivation at 85°C for 5 minutes. This

procedure was done for both the target gene (S1PR-1) and the reference gene (β -actin) and the various parameters displayed, including the melt curve as well as the various Ct scores, printed and used to calculate the gene expression fold for S1PR-1 in lymphocytes.

2.7.4 Real Time Polymerase Chain Reaction

The synthesized cDNA was amplified using the My IQ single color real time machine. The qPCR mix used was Solis Biodyne 5x HOT FirePolqPCR supermix plus. The reaction was done in 25 μ l reactions consisting of 4 μ l of the 5x HOTFirepolqPCR Mix, 0.4 μ l each of the forward primer 5'- CCGCTTGAG CGAGGCTGCTG-3' and reverse primers 5-CTATGA TATCATAGTTGCCATAGTC-3' and specific probe which had a concentration of 250nM, 18.2 μ l of Nuclease free water and 2 μ l cDNA template(100ng). A reference gene in this case β -actin was evaluated using forward primer 5'-CCTTCTACAAATGA GCTGCGT-3' and reverse primer 5'-CCTGGATAG CAACGTACATG-3'. All primers and kits were manufactured by Solis Biodyne chemical company, Tartu, Estonia. The cycling conditions were as follows; Initial Activation at 95°C for 12 minutes, Denaturation at 95°C for 15 seconds, Annealing at 48 and 51°C for 20 seconds (for SIPR-1 and β -actin respectively) and Elongation at 72°C for 20

seconds. The threshold cycle (Ct) obtained for the target gene (S1PR-1) and the house keeping gene (β -actin) were utilized in the calculation of the mRNA expression of S1PR-1 expression fold changes were calculated and presented as chart.

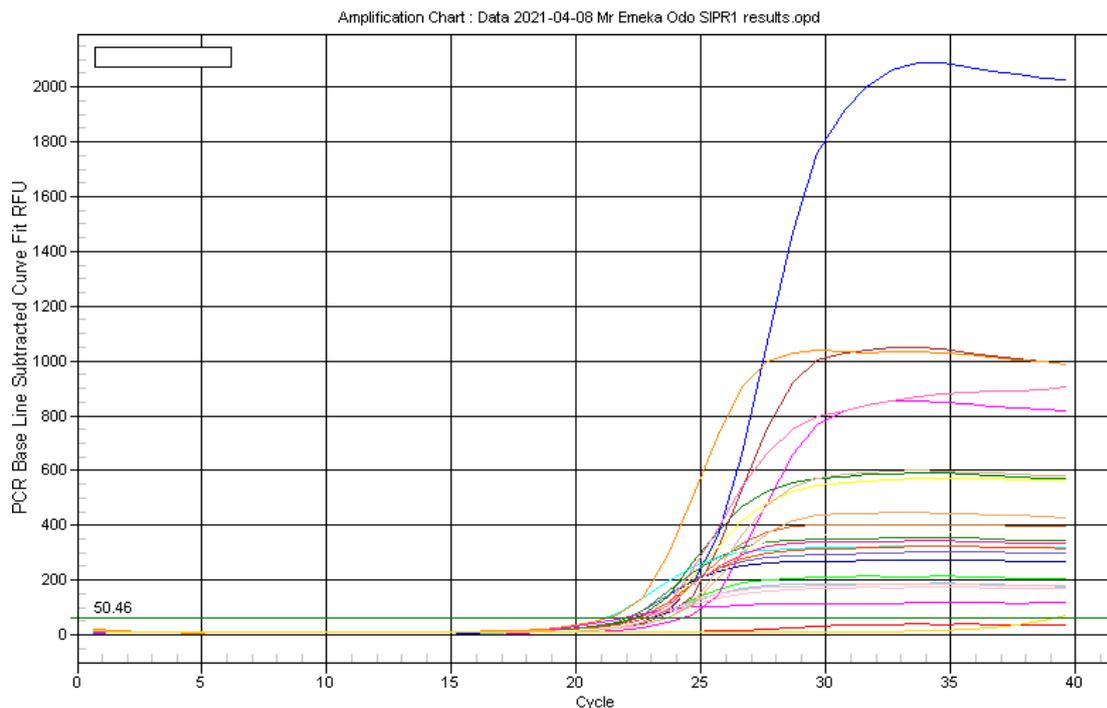
2.7.5 Data Analysis

The S1PR-1 expression fold was calculated by the method of $\Delta\Delta$ Ct, the student t-test/Z-test, Analysis of Variance (ANOVA) and Post- HOC multiple comparison (LSD) all of SPSS (statistical package for social sciences) was used to analyze the data and inferences made.

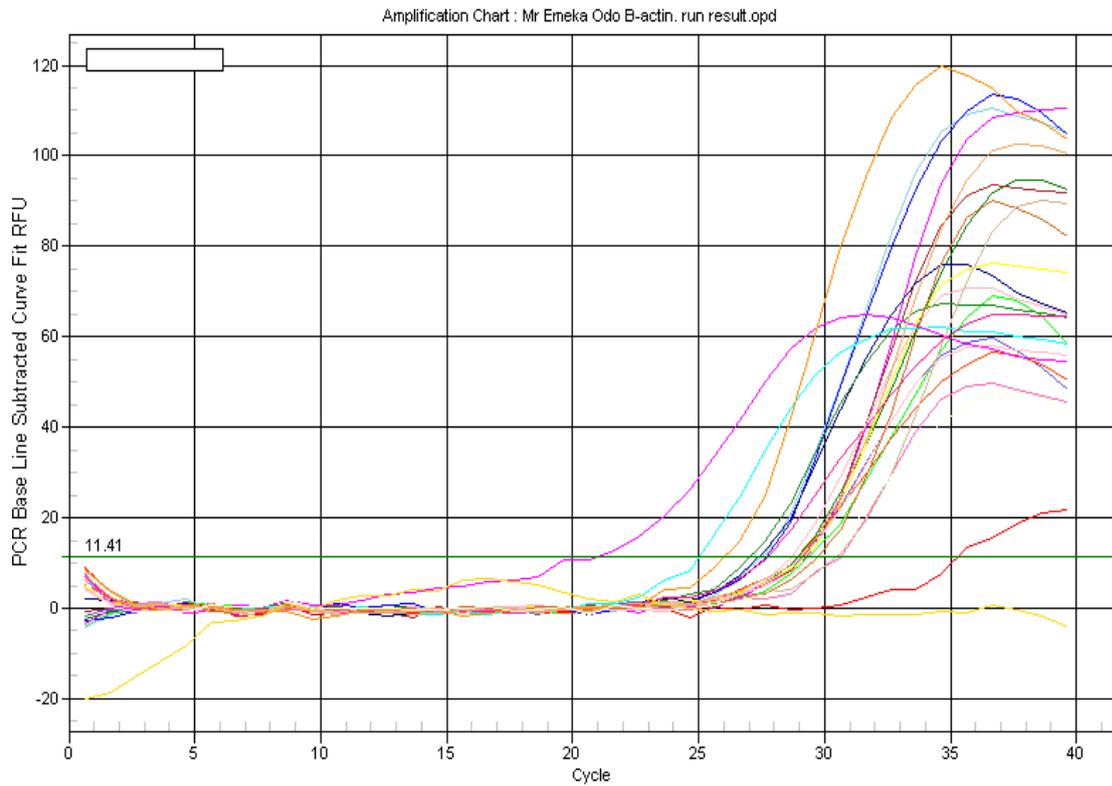
3. RESULTS AND DISCUSSION

The Fig. 1 indicate the expression fold of S1PR-1 in lymphocytes of the induced and treated groups when compared to the non-induced group 1 and 2.

The result indicates that the gene (S1PR-1) was downregulated in the experimental leukemia induced groups (3 to 9) of rats when compared to group 1 and 2. Conversely, after treatments, the downregulated gene (S1PR-1) was found to be upregulated across the respective extracts of *Hydrocotyle verticillata*, *Laportea aestuans* and the standard anti-leukemic drug treated groups.



Graph 1. Amplification plot SIPR-1



Graph 2. Amplification plot β -Actin

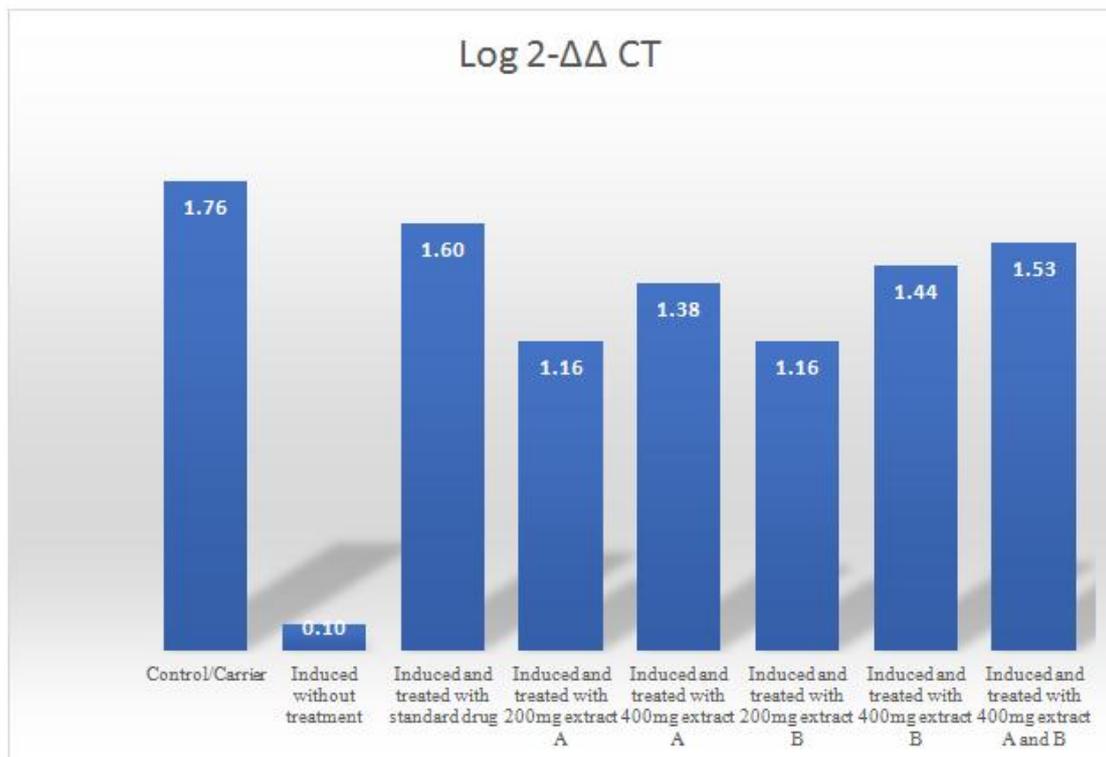


Fig. 1. Expression fold of S1PR-1 after induction and treatment of leukemia with extracts of *H. verticillata* and *L. aestuans*

Table 1. Total RNA yields of lymphocytes (after RNA extraction using Genzol RNA extraction kit) of rats after induction and treatment of leukemia with ethanol extracts of *H. verticillata* and *L. aestuans*.

S/N	Group	Total RNA concentration (ng/ml)
1	1	81.21
2	2	79.28
3	3	77.44
4	4	80.79
5	5	85.33
6	6	83.81
7	7	85.50
8	8	81.33
9	9	79.21

This upregulation was high with the standard drug, followed by the group with combined dose of 400mg of both extracts, then 400mg of *Laportea aestuans*, 400mg of *Hydrocotyle verticillata*, while 200mg of each of the extract of *Hydrocotyle verticillata* and *Laportea aestuans* was upregulated in the same manner.

The use of natural products obtained from plants extracts for pharmacological applications has continued to culminate in the discovery and development of natural and less-toxic anticarcinogenic agents that are promising at ameliorating the challenges of neoplastic diseases such as leukaemic cancer. In fact various beneficial biological activity such as anticancer, antimicrobial, antioxidant, antidiarrheal, analgesic and wound healing activities of medicinal plants and plant products have been reported. It is therefore suggestive to say that the comprehensive scientific investigations of the anti-leukemic potentials of some herbs, *Laportea aestuans* and *Hydrocotyle verticillata*, from our locale considering the huge anecdotal claims on them may be of great benefits. Of course, the discovery of any possible anti-leukemic effects of the ethanolic extracts of *Laportea aestuans* and *Hydrocotyle verticillata* may present them as possible ready sources of natural, safer, readily available and cheaper leukemic remedies for the mammalian living system. Therefore, the evaluation of the roles of ethanol extracts of *Laportea aestuans* and *Hydrocotyle verticillata* in the regulation of mRNA expression of S1PR-1 in experimentally induced leukemic conditions in wistar rats was done by the current study and the discussion of findings of the evaluation are presented below.

Considering this interesting outcome of the present study, it is important to state that our study has, for the first time, provided a scientific

explanation to the anti-leukemic potentials of the ethanolic leaf extracts of *L. aestuans* and *H. verticillata* plants. And the basic molecular site and mechanism of action is on S1PR-1 gene at the DNA (deoxyribonucleic acid) and regulation of its production of mRNA (messenger ribonucleic acid) in lymphocytes.

It is important to note that the levels of mRNA are considered as equal with S1PR-1 expression fold at the protein level, and in some types of cells, this similarity may not be so visible; however, for all the studies done on S1PR-1 in different kinds of cells, there were no reports having alternative view between S1PR-1 expression at the level of protein and mRNA level. S1PR-1 plays a pertinent role in T- and B-lymphocyte movements and propel their entry from the lymph nodes and thymus into the circulation [22,23]. S1PR-1 is thought to regulate naive lymphocyte egress from the lymph node through the lymphatic vessels by closing the lymphatic endothelial barrier and preventing naive T cell entry from lymph node and thymus. Noteworthy is that S1PR-1 was found to be expressed at variable levels on leukemic cells in leukemia of chronic lymphocytic origin [3]. It is important to note that the receptor encountered by leukemic cells within the tissue space might have resulted in the reduction of the mRNA expression of S1PR-1 [23]. It was suggested that leukemic cells could reduce the expression of S1PR-1 and this may delay their entry or movement into the circulation, thereby prolonging their stay at survival niches [23]. S1P has been implicated in disorders such as cancer and inflammatory diseases, and several agonists and antagonists have been developed and are in clinical trials that target S1PR-1 [24]. It is possible that the various phytochemicals in the plants (*Hydrocotyle verticillata* and *Laportea aestuans*) contains some S1PR-1 agonists/antagonists that regulate

S1PR-1 expression and the number of lymphocytes in the DMBA treated rats.

Recall that signaling of S1P through its cell surface receptor S1PR-1 has been identified to play an important role in carcinogenesis, cancer growth and survival, and tumor metastasis [4]. S1PR-1 is the receptor for the molecule S1P that controls the exit of lymphocytes from the lymph node. S1P signaling is a key regulator of T cell exit from both lymphoid and non-lymphoid tissues, as well as T cell retention in tissue microenvironments such as the germinal center [5]. The consequences of a downregulated S1PR-1 could imply poor dysregulation of T-Cell maturation and subsequent advent of a leukemic conditions which was upregulated upon administration of the various ethanolic extracts of the plants as well as the standard anti-leukemic drug. This upregulation by the various ethanolic extract of the plants could be attributed to the various phytochemicals that abound in these plants which have been found to exert anti-cancer, cytotoxic as well as anti-oxidants effects respectively [25].

This finding of the present study is consistent with the major finding of an earlier study which stated that the effect of a four-plant mixture (*Arctium lappa*, *Ulmus rubra*, *Rumex acetosella*, and *Rheum palmatum*) decreased the percentage of blasts by two thirds in leukemic rats and that the sphingosine-1-phosphate receptor-1 mRNA expression in lymphocytes was downregulated in leukemic rats they used [3].

5. CONCLUSIONS

The plant extracts have proven to reverse the down-regulation of mRNA expression of S1PR-1 in a leukemic rat model. The extracts particularly regulated the gene (S1PR-1) by up-regulation of experimentally down-regulated gene (S1PR-1); thus, implying that the plant extracts are potent anti-leukemia agents. This outcome of the present study further validates some earlier reports [3,25,26] on anti-carcinogenic properties of some plants extracts and reveals the possibility of their use as adjunct in leukemia treatment in order to prevent some negative side effects of the usual therapy.

CONSENT

It's not applicable.

ETHICAL APPROVAL

Ethical approval for the present study was sought for and obtained from the Ethics Committee of

Centre for Research Management and Development (with reference number: UPH/CEREMAD/REC/04, dated August 1, 2021), University of Port Harcourt, Nigeria. As well, all animals were handled according to the recommendations of the US National Institute of Health (NIH) guidelines for care and use of laboratory animals in experimental research (National Research Council, 2011).

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CONFLICTS OF INTERESTS

The authors hereby confirms that this article contents have no conflicts of interest. Though a lot of products were used in the study but we state here that no conflicts of interest between the manufacturers of the products and the authors as this is mainly for dissemination of knowledge.

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