Study of the antioxidant and immunomodulatory activity of the total aqueous extract of *Clerodendrum splendens* (G.Don) leaves (Lamiaceae)

ABSTRACT

Clerodendrum splendens (Lamiaceae) is a plant with therapeutic properties, traditionally used in several countries for the treatment of several diseases. The objective of this study was to evaluate the antioxidant and immunomodulatory activity of the total aqueous extract obtained from the leaves of *C. splendens* in order to contribute to a better knowledge of the therapeutic effects of this plant. It consisted in the realization of the antioxidant activity of ETAC according to the following methods: the DPPH test and the iron reducing power test (FRAP). The results of the study showed that ETAC has a good free radical scavenging activity compared to the activity of BHT, which is a synthetic antioxidant used as a standard. ETAC significantly increased white blood cell and lymphocyte counts at 125 and 250 mg/kg bw. The increase was greatest at 250 mg/kg bw. Only at 250 mg/kg bw was there a significant increase in neutrophils. Histological sections showed no structural or functional abnormalities in the thymus and spleen.

Keywords: Antioxidant activity, *Clerodendrum splendens*, Immunomodulator, Leukocyte parameters

1. INTRODUCTION

Medicinal plants are the best sources of medicines according to the World Health Organisation (WHO). Indeed, nearly 60% of the world's population uses herbal medicines as a pharmaceutical remedy. In addition, 80% of the population in Africa still uses traditional medicine to meet their health care needs. This shows that plants continue to play an important role in the maintenance of people's health, since ancient times [1]. Moreover, they are still an essential raw material in the manufacture of many drugs [2]. Indeed, medicinal plants produce a wide range of different bioactive molecules that are involved in the development of new drugs. Therefore, such plants should be studied to better understand their properties and efficacy [3]. The study of plant chemistry is still relevant today, despite its long history. This is mainly due to the fact that the plant kingdom represents an important source of basic medicines. The Ivorian territory, due to its geographical position, presents a wide range of rich vegetation and various bioclimatic stages of plants used as condiments, natural foods and for therapeutic

purposes. Among these medicinal plants, those belonging to the genus *Clerodendrum* (Lamiaceae), are widely used in the treatment of various diseases, chronic and acute disorders. Extracts obtained from the roots, leaves and bark of *Clerodendrum splendens* are used to treat malaria, coughs, venereal infections including gonorrhoea and syphilis, skin diseases, ulcers, rheumatism, asthma, and uterine fibroids [4; 5 and 6]. Most of the plant species growing around the world have therapeutic properties, as they contain active ingredients that act directly on the body. However, the geographical location of a plant may affect its active component. A plant growing wild in one country may not necessarily have the same components as the same plant in another country, and their biological activity may not be similar. This is why we undertook the study of some pharmacological activities of *C. splendens* from the lagoon region. Considering the immunomodulatory effect of some *Clerodendrum* species, the antibacterial effect of *C. splendens* extract [7 and 8], as well as the rich content of this plant in secondary metabolites and its anti-inflammatory effect [4 and 5], we oriented our study to verify a possible antioxidant and immunomodulatory effect of the total aqueous extract of *Clerodendrum splendens* (Lamiaceae) leaves, with a view to contributing to the valorisation of this species.

2. MATERIAL AND METHODS

2.1. Material

2.1.1. Plant material

The plant material consists of *Clerodendrum splendens* (Lamiaceae) leaves collected in June, in the SICOGI 1 district, on the Bingerville-Abidjan axis (Côte d'Ivoire).

2.1.2. Animal material

White albino male and female Wistar rats were used for the acute and subacute toxicity study. These animals come from the animal house of the Life and Earth Sciences Laboratory of the Normal School Superior (ENS) in Abidjan. They were kept under standard lighting conditions (12 hours of light, 12 hours of darkness) at an ambient temperature of 25 ± 1 °C. These animals had free access to standard food and tap water.

2.2. Methods

2.2.1. Preparation of the extract

The collected leaves of *Clerodendrum splendens* (Lamiaceae) were cut into small pieces and dried at room temperature for two (2) weeks. After drying, they were ground to obtain a fine plant powder. The total aqueous extract was prepared according to the method described by Guédé-Guina [9] with slight modifications. Thus, 50 grams of plant powder were dissolved in

one litre of distilled water. The aqueous mixture was stirred with a Blinder-type mixer for three minutes and the operation was repeated three times. The resulting homogenate was filtered three times on square cloths. The filtrate obtained was purified four times on cotton wool and once on Whatman paper (3 mm). The new filtrate was evaporated at 50°C using an oven (Memmert, Germany) to obtain the total aqueous extract of *C. splendens* (ETAC).

2.2.2. In vitro evaluation of the antioxidant activity of the total aqueous extract of *C.splendens* leaves (ETAC)

2.2.2.1. DPPH test

The free radical scavenging activity of *C. splendens* extract was determined using DPPH as a relatively stable free radical according to the protocol described by Boumerfeg [10]. In this assay, antioxidants reduce diphenyl picryl hydrazyl (DPPH) having a purple colour to a yellow compound. This allows to follow the kinetics of decoloration at 517 nm. Thus, fifty (50) µl of each of the different concentrations of the extract were incubated with 5 ml of a 0.004% methanolic solution of DPPH. After an incubation period of 30 minutes, the absorbances at 517 nm were recorded. The results obtained for the tested extract were expressed in relation to those obtained for BHT taken as reference antioxidant. The anti- radical activity is estimated according to the equation below:

$$\% \text{ of anti-radical activity} = \frac{(\text{A517 control} - \text{A517 sample})}{\text{A517 control}} \times 100$$

The 50% inhibitory concentration of DPPH activity (IC_{50}) expressed in µg/ml of each extract is calculated and compared with that of BHT.

2.2.2.2. Reducing power test (FRAP)

The FRAP (Ferric Reducing/Antioxidant Power) test was performed according to the method originally described by Benzie and Strain [11] and modified by Pulido [12]. This method measures the reducing power of antioxidants in a mixture by their ability to reduce ferric tripyridyl-triazine (Fe^{3+} -TPTZ) to ferrous (Fe^{2+}-TPTZ) at acid pH. A fresh solution of the FRAP reagent was prepared by mixing 2.5 ml of the TPTZ solution (10 mM in 40 mM HCl) with 2.5 ml of $FeCl_3.6H_2O$ (20 mM) and 25 ml of acetate buffer (300 mM sodium acetate, pH brought to 3.6 by acetic acid). Nine hundred (900) µl of the FRAP reagent, previously incubated at 37°C, was mixed with 70 µl of doubly distilled water and 30 µl of the sample

(with appropriate dilutions). The increase in absorbance at 593 nm was then monitored for 30 minutes at 37 °C. A range of $FeSO_4.7H_2O$, a reducing standard, between 0 and 2000 µM was used to calculate the FRAP values of the extracts and the standard antioxidant (BHT). The EC1 parameter (equivalent concentration 1) of the extracts and standard was calculated at 4 and 30 minutes. EC1 is defined as the concentration of antioxidant that gives a reduction in TPTZ equivalent to 1 mM $FeSO_4.7H_2O$; it is the concentration of antioxidant that gives an increase in absorbance at 593 nm equivalent to the theoretical value of absorbance given by a concentration of 1 mM $FeSO_4$ determined by the corresponding regression equation. The total antioxidant power (TAP) of extracts and standard was expressed in micromoles of $FeSO_4.7H_2O$ equivalents per mg of extract (µmole Eq $FeSO_4.7H_2O$/mg).

2.2.3. Immunomodulatory effect of ETAC

The OECD (2008) guideline 407 for the testing of chemicals, with some modifications, was used for this study. Thirty-two (32) rats aged eight (8) to twelve (12) weeks with weights ranging from 150 to 200 grams on average were used. The rats were randomly divided into four (4) batches of eight (8) rats each according to their weight. Each batch consisted of four (4) male and four (4) female animals divided into different bins according to sex. In each batch, the number and sex of the animals were marked on the bins. The rats in the control lot were each given 1 ml of distilled water by gavage daily during the 28-day treatment period. The three (3) test batches (batches 1; 2 and 3) received 125 mg/kg bw; 250 mg/kg bw and 500 mg/kg bw of ETAC daily by gavage for 28 days. The volume of ETAC administered daily as a single dose was 1 mL. During the 28 days of treatment, animals were observed daily for clinical signs and symptoms of toxicity before, immediately and three (3) hours after ETAC administration. On the 29th day, the day after the last day of treatment, all rats were sacrificed by the decapitation technique. In this technique, after anaesthetising the rats with ether, their necks were cut with sterile cutting scissors. After each section, the blood was immediately collected in dry tubes and EDTA (ethylene-diamine-tetra-acetic acid) tubes for the following respective analyses: biochemical and haematological. Organs such as the thymus and spleen were removed, cleaned with saline and weighed. These organs were preserved in 10% formalin for histological sections.

2.2.4. Determination of haematological parameters

The haematological analysis was performed using an automatic analyser (URIT®-2900 PLUS) which gave direct values for the following leucocyte parameters: white blood cell (WBC) count, monocytes, neutrophils, eosinophils and lymphocyte percentage.

2.2.5. Determination of biochemical parameters

The blood in the dry tubes was centrifuged using a centrifuge at 3000 rpm for 5 minutes. The serum obtained was collected and stored at -20°C for serum marker analysis using the ROBONIK® PRIETES TOUCH analyser.

2.2.6. Histopathology examination

For histopathology examination, the organs underwent a series of dehydration in ethanol baths, and inclusion in paraffin. Sections of 5µm were made with a microtome and then stained with Haematoxylin and Eosin (H&E) and observed under a light microscope (Olympus CKX41, Germany) [13].

2.2.7. Statistical analysis

Statistical analysis and graphical representations were carried out using Graph Pad Prism version 8.02. The values are presented as mean ± standard deviation. The comparison of means and variances was carried out by the ANOVA test followed by the Tukey test.

3. RESULTS AND DISCUSSION

3.1. Results

3.1.1. In vitro evaluation of the antioxidant activity of ETAC

3.1.1.1. DPPH test

The results indicate that the percentage of DPPH free radical inhibition increases with increasing concentration of butylated hydroxytoluene (BHT) and total aqueous leaf extract of *C. splendens* (ETAC). The antioxidant activity of the extract and the standard is expressed as IC_{50}, which represents the effective concentration of substrate required to cause 50% loss of DPPH activity. This concentration is reached respectively at 0.988 mg/ml for ETAC and 0.904 mg/ml for BHT (standard). The difference between these two concentrations remains insignificant (Figure 1).

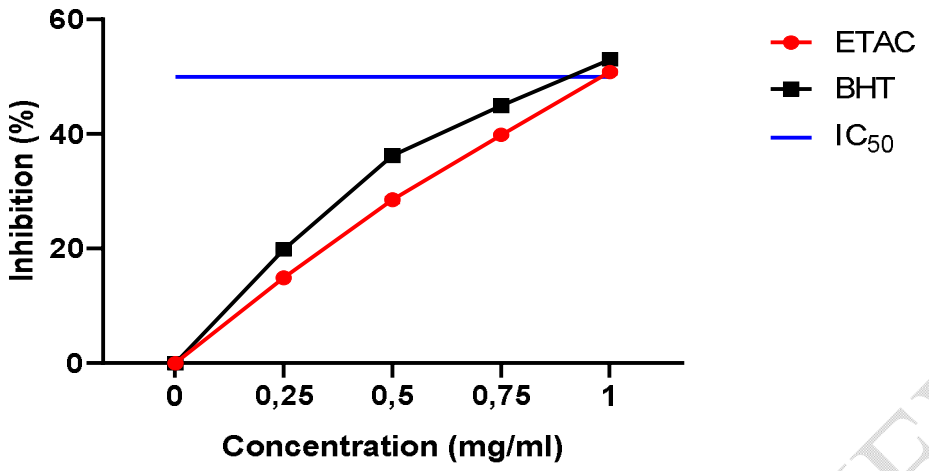

Figure 1: Percentage DPPH radical inhibition of the reference antioxidant and ETAC

ETAC: Total aqueous leaf extract of *C. splendens*, **BHT:** butylated hydroxytoluene, **IC_{50}:** 50% inhibitory concentration of DPPH activity

3.1.1.2. Reducing power test (FRAP)

Figure 2 shows the iron reduction capacity (reducing power) of ETAC and that of the standard (BHT). The results obtained show that the capacity of BHT to reduce iron is much higher than that of the extract. The extract exerts a lesser reducing power with IC_{50} which is reached at 0.350 mg/ml compared to that of BHT which is reached at 0.113 mg/ml.

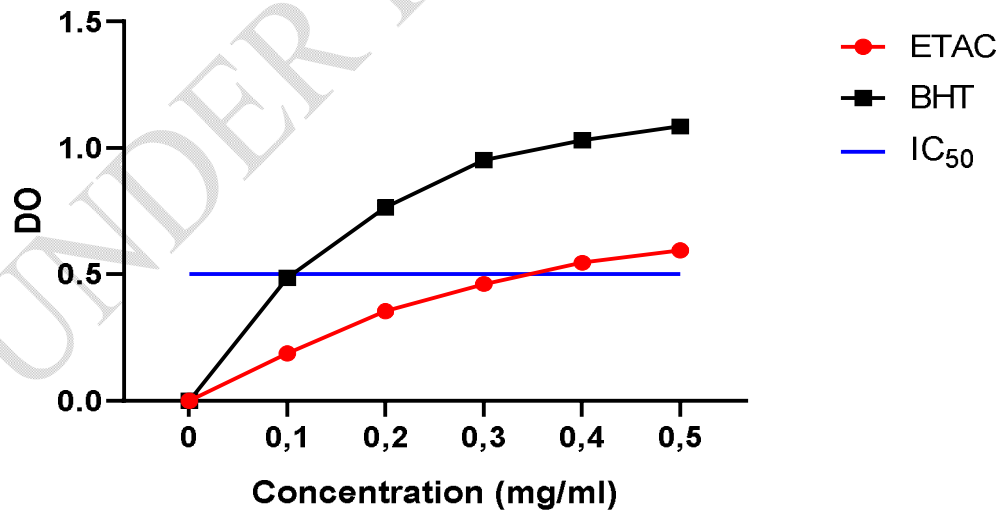

Figure 2: Reducing power of the reference antioxidant and ETAC

ETAC: Total aqueous leaf extract of *C. splendens*, **BHT:** butylated hydroxytoluene, **IC_{50}:** 50% inhibitory concentration of DPPH activity

3.1.2. Effect of ETAC on leukocyte parameters

White blood cells (WBC), lymphocytes, monocytes, neutrophils, eosinophils and basophils were determined. The effect of total aqueous extract of *C. splendens* (ETAC) on these leukocyte parameters in treated and control rats is presented in Table I. The administration of 500 mg/kg bw for the 28 days did not have a significant impact on these parameters compared to the control lot ($p > 0.05$). Basophil values showed a significant increase ($p < 0.001$) from 17.60 ± 0.49% (control value) to 22.98 ± 0.33 (500 mg/kg bw value). At 125 mg/kg bw, there was an increase in white blood cell values, but the increase was non-significant ($p > 0.05$) from 12.27 ± 0.36 ×103/µL (control) to 13.57 ± 0.48 ×103/µL (125 mg/kg bw). The 125 mg/kg bw dose resulted in a significant ($p<0.001$) increase in lymphocyte values from 12.69 ± 0.25% (control) to 14.64 ± 0.25% (125 mg/kg bw). It also resulted in a significant increase ($p<0.01$) in basophil values from 17.60±0.49% (control) to 21.02±0.87% (125 mg/kg bw) and in monocyte values from 2.125±0.09% (control) to 2.480±0.09% (125 mg/kg bw) at $p<0.05$. All three doses, 125, 250 and 500 mg/kg bw, resulted in an increase in eosinophil values. However, this increase, which was insignificant ($p > 0.05$), increased from 1.19±0.06% (control value) to 1.20±0.04%, 1.21±0.05% and 1.22±0.07% respectively for the 125 mg/kg bw, 250 mg/kg bw and 500 mg/kg bw doses. The 250 mg/kg bw daily dose resulted in a significant ($p<0.0001$) increase in lymphocyte values from 12.69 ± 0.25 ×103/µL (control) to 18.93 ± 0.14 ×103/µL (250 mg/kg bw) and in Monocyte values from 2.125 ± 0.09% (control) to 2.988 ± 0.06% (250 mg/kg bw). This dose also resulted in a significant increase ($p<0.001$) in White Blood Cell values from 12.27 ± 0.36% (control) to 15.24 ± 0.45% (250 mg/kg bw) and Basophil values from 17.60 ± 0.49% (control) to 22.06 ± 0.74% (250 mg/kg bw). However, the 250 mg/kg bw dose resulted in a significant ($p<0.01$) increase in neutrophil values from 56.35 ± 0.36% (control) to 60.74 ± 1.10% (250 mg/kg bw).

Table I: Leukocyte profile of rats treated with different doses of oral ETAC for 28 days

	Batches			
	Distilled water (control)	**ETAC 125 (mg/kg bw)**	**ETAC 250 (mg/kg bw)**	**ETAC 500 (mg/kg bw)**
White blood cells (×10^3/µL)	12,27 ± 0,36	13,57 ± 0,48 **ns**	15,24 ± 0,45 ***	11,97 ± 0,13 **ns**
Red blood cells (×10^6/µL)	7,02 ± 0,18	6,95 ± 0,21 **ns**	7,19 ± 0,13 **ns**	7,10 ± 0,17 **ns**
Lymphocytes (%)	12,69 ± 0,25	14,64 ± 0,25 ***	18,93 ± 0,14 ****	13,47 ± 0,65 **ns**
Monocytes (%)	2,125 ± 0,09	2,480 ± 0,09 *	2,988 ± 0,06 ****	2,067 ± 0,08 **ns**
Neutrophils (%)	56,35 ± 0,36	59,04 ± 0,61 **ns**	60,74 ± 1,10 **	59,82 ± 0,80 **ns**
Eosinophils (%)	1,19 ± 0,06	1,20 ± 0,04 **ns**	1,21 ± 0,05 **ns**	1,22 ± 0,07 **ns**

Basophils (%)	17,60 ± 0,49	21,02 ± 0,87 **	22,06 ± 0,74 ***	22,98 ± 0,33 ***

The values are means ± standard errors, each batch consists of 8 animals (n=8/batch). Statistical analyses are done per line compared to the control lot.

ETAC: Total aqueous leaf extract of *C. splendens*; **ns:** There is no significant difference at $p > 0.05$; *: Significant difference at $p < 0.05$; **: Very significant difference at $p < 0.01$; ***: Very very significant difference at $p < 0.001$; ****: Highly significant difference at $p < 0.0001$.

3.1.3. Histology of lymphoid organs (thymus and spleen)

Observation of thymus sections from rats of different batches revealed the presence of a faintly stained inner zone called the medullary zone, surrounded by a dense outer layer, the cortical zone (figure 3). The cortex is surrounded by a capsule of connective tissue from which short septa containing blood vessels extend deep into the cortex. These different structures are present in both controls and treated patients. ETAC had no effect on this organ.

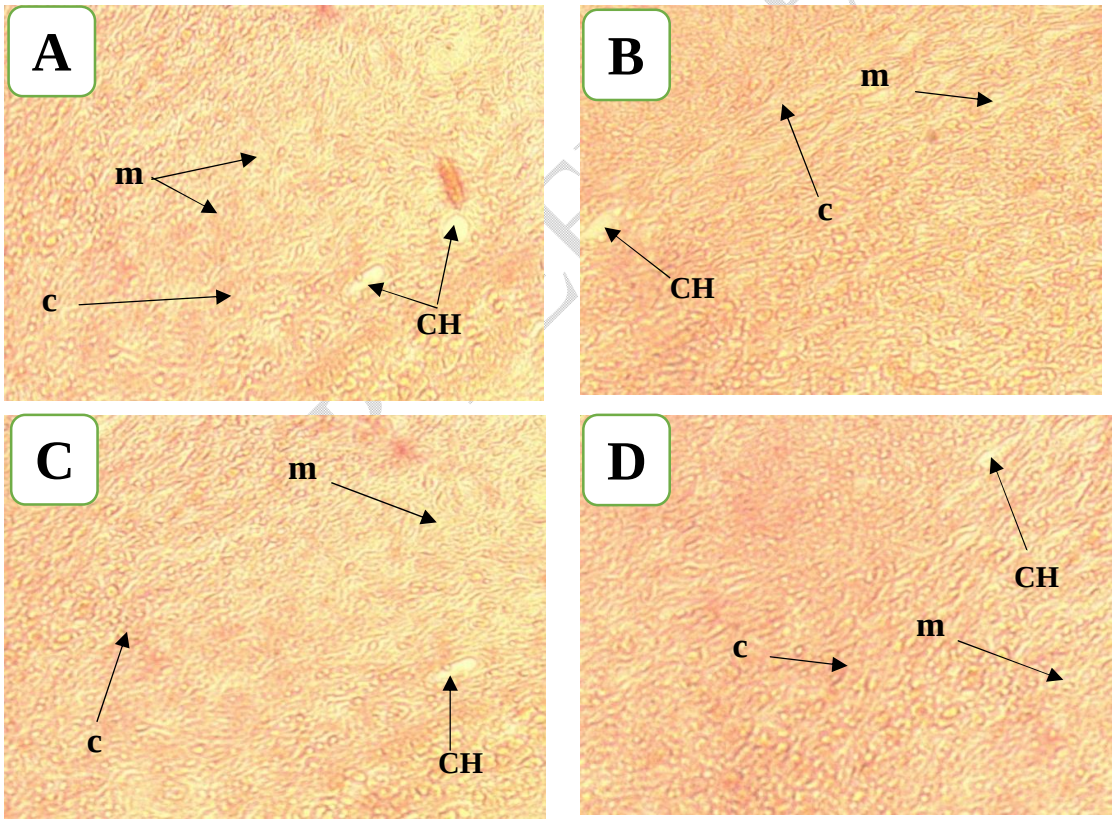

Figure 3: Histological sections of thymus from treated rats

Hematoxylin and Eosin; G× 100

A: control rat thymus, **B:** rat thymus treated with 125 mg/kg bw ETAC, **C:** rat thymus treated with 250 mg/kg bw ETAC, **D:** rat thymus treated with 500 mg/kg bw ETAC, **CH:** Hassal's corpuscle; **m:** medullary zone; **c:** cortical zone

The cross-sections of the spleens of rats from the different batches showed small whitish nodules, called white pulp (lymphoid tissue), surrounded by a red matrix, the red pulp. The white pulp is made up of lymphocytic aggregates and the red pulp, which makes up the bulk of the organ, is a richly vascularised tissue (figure 4).This organ was not damaged.

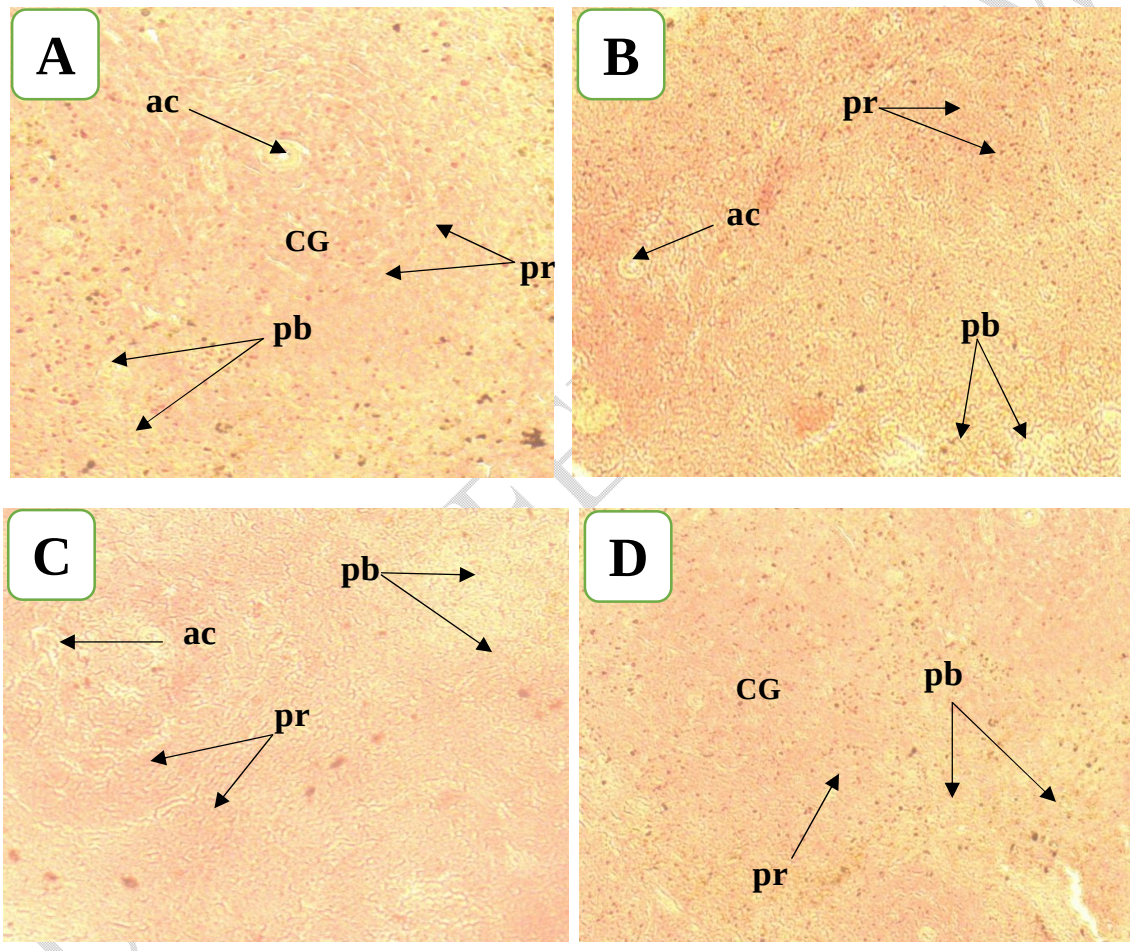

Figure 4: Histological sections of the spleen of treated rats

Hematoxylin and Eosin; G× 100

A: rat spleen control, **B:** rat spleen treated with 125 mg/kg bw ETAC, **C:** rat spleen treated with 250 mg/kg bw ETAC, **D:** rat spleen treated with 500 mg/kg bw ETAC

CG: germinal centre; **ac:** corpuscular artery; **pb:** white pulp; **pr:** red pulp

3.2. Discussion

The study concerned the antioxidant and immunomodulatory activity of the total aqueous extract of *Clerodendrum splendens* (ETAC) leaves (Lamiaceae). The choice of this plant is explained by the multiplicity of therapeutic virtues attributed to it. The leaves were chosen as plant material because they are the most widely used in the traditional treatment of many diseases.

The antioxidant activity of ETAC is determined by two methods: the DPPH test and the iron reducing power (FRAP) test. These methods serve as significant indicators of the potential antioxidant activity of the compounds. The results of the study showed that ETAC has a good free radical scavenging activity compared to the activity of BHT, which is a synthetic antioxidant, used as a standard. The phenolic compounds contained in the extract are probably responsible for the antioxidant activity [14]. Indeed, molecules such as ascorbic acid, tocopherol, chlorogenic acid, quercetin, kaempferol derivatives and tannins have been shown to reduce and decolorise DPPH due to their ability to release hydrogen [15 and 16]. Furthermore, it is well established that antioxidant activity is positively correlated with the structure of polyphenols. Generally, polyphenols with a high number of hydroxyl groups show higher antioxidant activity [17] due to their ability to donate more atoms to stabilize free radicals [18]. The results of the present study are far superior to those obtained by Nam [19] who worked on ripe papaya pulp. These authors reported IC_{50} s ranging from 2.83 mg/mL to 11.90 mg/mL depending on the type of extraction solvents used. The reducing power (FRAP test) expresses the ability of a compound to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) by electron transfer. The results of our analysis revealed that ETAC has a low reducing capacity compared to BHT. The reducing power is probably due to the hydroxyl groups of the phenolic compounds in the extract which can serve as electron donors. The reducing power values determined in the present study are much higher than those reported by Sultana [20] who worked on the kernels and peelings of two mango varieties (Langra and Chonsa). Indeed, these authors reported reducing powers at a concentration of 10 mg/mL of between 0.595 and 0.624 respectively for peels and kernels of the Chonsa variety and between 0.607 and 0.850 for those of the Langra variety.

Concerning the leukocyte parameters, it was revealed that the 125 and 250 mg/kg bw doses resulted in an increase in white blood cell and neutrophil values. This increase was highly significant ($p < 0.001$) with the 250 mg/kg bw dose. However, it was insignificant ($p > 0.05$) with the 125 mg/kg bw dose. These doses (125 and 250 mg/kg bw) resulted in a significant increase in lymphocytes, monocytes and basophils. This increase was greater ($p < 0.0001$) with

the 250 mg/kg bw dose. In contrast, the 125 and 250 mg/kg bw doses resulted in a significant decrease in eosinophil counts compared to controls. This decrease was more marked ($p < 0.0001$) with the 250 mg/kg bw dose compared to controls. Indeed, the increase in white blood cells is a defence reaction of the body against cancer of the bone marrow cells (leukaemia), bacterial infections and parasitic infections. White blood cells ensure the sanctity of the body [21]. An increase in lymphocytes would indicate the presence of an inflammatory reaction. They are specific to the infectious agents present and form part of the body's first line of defence. The stimulation of the inflammatory reaction by these doses (125 and 250 mg/kg bw) would be due to the presence of flavonoids and saponosides in ETAC [22]. The increase in neutrophil levels at the 250 mg/kg bw dose is thought to contribute to the strengthening of non-specific antibacterial defence, thanks to their properties of displacement (chemotaxis), engulfment (phagocytosis) and extinction of bacterial life (bactericide). This increase would also be due to the presence of flavonoids which have an important role in the treatment of inflammation. Flavonoids stimulate anti-inflammatory activities without having ulcerogenic side effects [23]. The high presence of monocytes (250 mg/kg bw) is thought to be due to their residence (half-life about 2-3 days) in the blood before migrating into the tissues (bone marrow, spleen, lymph nodes, connective tissues, subcutaneous tissues, lungs) where they differentiate into macrophages.

At the same dose, the eosinophil count increased compared to controls. But this increase was insignificant. Eosinophils play an important role in the immune system. They are mainly involved in the destruction of parasites via high molecular weight proteins or in allergic reactions. The immunomodulatory properties of these doses (125 and 250 mg/kg bw) might suggest the presence of type II arabinogalactans. These type II arabinogalactans may be one of the main structures responsible for macrophage activation although the presence of type I arabinogalactans in these doses could not be excluded. Therefore it would be necessary to determine the structure and nature of the polysaccharide molecule responsible for the immunomodulatory activities of these doses [24]. This molecule, the most active of the strong immunomodulatory activity, is believed to stimulate the production of cytokines. These cytokines consist of the provocative cytokines IL 1b, IL 6, TNF and GM-CSF, and the anti-inflammatory cytokines IL 10. These polysaccharides appear to be unique in their ability to enhance GM-CSF production. In general, the polysaccharide fractions contain polyphenols over a range of concentrations. Polysaccharides are able to bind to identified polyphenols through intermolecular interactions, changing the molecular conformation of carbohydrates [24].

In contrast, the 500 mg/kg bw dose used did not induce significant changes ($p > 0.05$) in leukocyte parameters such as white blood cell, lymphocyte, monocyte, neutrophil and eosinophil levels compared to the control lot. Only basophil values showed a significant increase ($p < 0.001$). This dose did not produce any alteration in the values of the leukocyte parameters. This indicates that the 500 mg/kg bw dose did not affect any immune cells. The statistically identical values for lymphocytes, monocytes, neutrophils and eosinophils in the 500 mg/kg bw dose and the control would indicate that the 500 mg/kg bw dose of ETAC is not immunogenic, thus unable to stimulate the immune system. Observation of histological sections of the thymus and spleen showed no structural changes in these organs in treated rats compared to controls.

4. CONCLUSION

At the end of this study, the analysis of the antioxidant activity shows that ETAC has a good free radical scavenging activity compared to the activity of BHT which is a synthetic antioxidant, used as a standard. ETAC has a low reducing capacity compared to BHT. The analysis of leukocyte parameters after 28 days of treatment of rats showed that 125 and 250 mg/kg bw of the total aqueous extract of the leaves of *Clerodendum splendens* (ETAC) has a remarkable immunostimulatory effect on white blood cells and lymphocytes. However, this stimulation is greater with the 250 mg/kg bw dose. Histological sections showed that ETAC had no adverse effects on the structure and function of the thymus and spleen.

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