

# Aqueous Leaves Extract of *Gongronema latifolium* (Benth) Downregulates the Expression of IFN- $\gamma$ , IL-10 and Cell Surface Markers in Rabbits

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**How to cite this paper:** Rowaiye, A.B., Njoku, M., Oli, A.N., Igbokwe, N.H., Asala, T., Salami, S.A., Uzochukwu, I.C. and Esimone, C.O. (2022) Aqueous Leaves Extract of *Gongronema latifolium* (Benth) Downregulates the Expression of IFN- $\gamma$ , IL-10 and Cell Surface Markers in Rabbits. *Open Journal of Medical Microbiology*, 12, 117-128. <https://doi.org/10.4236/ojmm.2022.124011>

**Received:** September 3, 2022

**Accepted:** October 28, 2022

**Published:** November 1, 2022

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## Abstract

**Background:** The pathophysiology of the inflammatory process reveals intricate signaling which includes the IL-1 $\beta$ , IL-6, and TNF $\alpha$  pathways that could serve as drug targets. **Aim:** This study determined the effect of the aqueous extract of *Gongronema latifolium* (AEGL) leaves on the expression of IFN $\gamma$ , IL-10, CD3, and CD56 in rabbits. **Materials and Methods:** ELISA tests were performed to determine the effect of the AEGL on the expression of a pro-inflammatory cytokine (IFN $\gamma$ ), an anti-inflammatory cytokine (IL-10), and CD3 and CD56 cell surface markers in rabbits. Twenty cross-bred male rabbits with an average weight range of 1.0 - 1.5 kg were selected. The rabbits were separated into four groups of four rabbits each treated as follows: Grp1 is the untreated control; Grp2 is the treated control; and Grp3, Grp4, and Grp5 were treated with 200, 400, and 600 mg/kg of AEGL respectively for 28 days. **Results:** The AEGL showed its greatest inhibitory effect in Group 4 on IL-10 (118.5 pg/ml), and IFN $\gamma$  (332 pg/ml) on days 14 and 21 respectively. AEGL also showed the highest inhibition of CD3 expression on days 14 and

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21 (0 pg/ml) in Group 3; and CD56 expression on day 21 (630.5 pg/ml) in Group 4. **Conclusion:** AEGL showed exhibited strong T cell mediated anti-inflammatory, and immunomodulatory activity in test rabbits within the 28-day period which can be confirmed by cell based assays. Specifically at 400 mg/kg, AEGL exhibited the greatest anti-inflammatory activity which is suggestive of its maximum effective dose.

### Keywords

Inflammation, Cell Surface Markers, Antioxidant Activity, Cytokine, Hepatic Toxicity, Medicinal Plant, *Gongronema latifolium*

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## 1. Introduction

Inflammation is an intricate biological process that involves the protection of the body from invading factors and at times marks the onset and/or the progression of diseases [1]. Inflammation involves a vast array of cells, cytokines, chemokines, acute phase proteins, cell surface markers, and immune mediators [2]. The inhibition of any specific protein in these cascades could serve as a viable drug target in the alleviation of inflammatory conditions. To maintain homeostasis, anti-inflammation is facilitated by certain cytokines and mediators and intricate signaling pathways [2]. In order to promote anti-inflammation, these immune agents must be activated. Food, herbs, drugs, and biologics offer a vast array of small molecules, peptides and proteins that have either pro- or anti-inflammatory activity [3].

The T Lymphocytes (T cells) and Natural Killer (NK) cells with CD3 and CD56 markers respectively are important players in the adaptive immune system. Through the production of cytokines and other immune mediators, they play crucial role in the process of inflammation and immunomodulation [4] [5]. IFN- $\gamma$  and IL-10 are key cytokines that alter the immune system with their pro-inflammatory and anti-inflammatory activities respectively [6]. The IL-10 or human cytokine synthesis inhibitory factor (CSIF) is a multifunctional cytokine that regulates immune and inflammatory responses. It is an anti-inflammatory cytokine which is released during systemic infections. It is produced by NKC cells, macrophages, dendritic cells (DC), B cells, and CD4+ T cells (Th2) [7]. IL-10 exerts pleiotropic effects on hemopoietic and non-hemopoietic cells such as by endothelial cells and keratinocytes [7]. It affects immunosuppression by the inhibition of the effector functions of NKC cells, macrophages, monocytes and Th1 cells. It does this by inhibiting the maturation and differentiation of these haemopoietic cells and subsequently their cytokines and chemokines [7] [8]. IL10 binds to and forms a complex with its receptors which consists of two IL-10R1 and two IL-10R2 molecules and the receptors so activated, trigger several signaling pathways which include the Jak1-Tyk2/Stat3 pathway [8].

IFN $\gamma$  inhibits the proliferation of Th2 cells and enhances the proliferation of

activated B cells. IFN- $\gamma$  is expressed by NK cells and is involved in tumor immune surveillance through cytostatic and cytotoxic mechanisms [9]. It could have either pro or anti tumorigenic activity depending on cellular or molecular microenvironment of the tumor [9] [10]. It also inhibits tumor development by providing protection through lymphocytes and by immunoediting of the cancer cell phenotype [7]. Due to the tumor-sculpting roles of immunity, IFN  $\gamma$  is been proven to be involved in cancer immunoediting and not cancer immunosurveillance [9].

Extracts of *Gongronema latifolium* (AEGL) has been reported to show anti-inflammatory and antioxidant activities [11]. This study is aimed at investigating the anti-inflammatory properties of AEGL through its effect on the expression of IFN $\gamma$ , and IL-10; and to trace the cellular sources of these cytokines through the expression of CD3, and CD56 in rabbits.

## 2. Materials and Methods

### 2.1. Experimental Animals

Twenty cross-bred male rabbits with an average weight range of 1.0 - 1.5 kg were selected. The 20 were reared at the Bioresources Development Centre, Isanlu (Kogi state, Nigeria). During the experimental period, the rabbits were housed in steel wired mesh cage measuring 50 cm  $\times$  50 cm  $\times$  40 cm at the well-ventilated Animal Facility Centre of the Nigerian Institute of Pharmaceutical Research and Development (NIPRD). Two weeks prior to the commencement of the study, the rabbits which were in good health were kept in the cages and no drugs were administered. The housing had a 12-hour light/dark cycle, a relative humidity between 36% - 40%, and a temperature of 28°C  $\pm$  2°C. The study rabbits were administered roughage, commercial grower pelleted feed, and water ad libitum. The design of the experiment and handling of the rabbits was in accordance with international standard [12].

### 2.2. Preparation of an Aqueous Extract of *Gongronema latifolium*

The leaves of the edible plant, *Gongronema latifolium* were obtained from a local farm in Ohafia, Abia state of Nigeria. The plant was taxonomically identified by the Herbarium of NIPRD and voucher number (NIPRD/H/6968) was issued. The leaves which had been dried at room temperature for 4 weeks was destalked and pulverized. For a period of 24 hours, 1.8 kg of dried *Gongronema latifolium* leaves was soaked in 24 L of water and agitated with a mechanical shaker (Karl Kolb Scientific Supplies, Germany). The solution was carefully filtered, and the filtrate was concentrated using a rotary evaporator (Bibby Sterlin Ltd., Staffordshire, England). The extract was stored at 4°C in a refrigerator. Using distilled water, different concentrations of the extract was prepared [13].

### 2.3. Experimental Design

The rabbits were separated into five groups of four rabbits each and treated as

follows: Group 1 is the Untreated Control (with no extract and no multivitamins); Group 2 is the treated Control (administered or treated with 0.33 ml of Multivitamins (EMVITE® Multivitamin Syrup) per kg body weight); Group 3 is treated with 200 mg/kg of AEGL; Group 4 is treated with 400 mg/kg of AEGL; and Group 5 is treated with 600 mg/kg of AEGL. Animals were weighed weekly and different doses of the aqueous extract of the plant were administered to the rabbits daily by intubation for 28 days.

#### **2.4. Sample Collection, Preparation and Testing**

Blood samples were collected from the lateral saphenous and ear veins of the rabbits at day 0, 7, 14, 21, and 28 of the administration of the AEGL. The plasma was obtained after centrifuging the blood at 2000 rpm for 10 minutes with Eppendorf Centrifuge 5702 (Eppendorf AG 22331, Hamburg, Germany).

The study samples and the IFN $\gamma$ , IL-10 and CD56 ELISA kits (Elabscience, China) were removed from the refrigerator and kept at room temperature for 20 minutes before the commencement of the experiment. 30 ml of Concentrated Wash Buffer (Elabscience, China) was diluted with 720 ml of deionized water (dilution factor of 25). 1 ml of Reference Standard & Sample Diluent (Elabscience, China) was added to the Standard (Elabscience, China) which had already been centrifuged for 1 minute. This standard working solution (1000 pg/ml) was allowed to stand for 10 minutes and diluted into different concentrations. For the dilution, the Reference Standard & Sample Diluent (Elabscience, China) was used to obtain 1000, 500, 250, 125, 62.5, 31.25, 15.63 and 0 pg/ml. To produce a Biotinylated detection Antibody (Elabscience, China) working solution, the Concentrated Biotinylated Detection Antibody (Elabscience, China) was centrifuged and diluted by a factor of 100 using the Biotinylated Detection Antibody Diluent (Elabscience, China). Also, to produce the Concentrated HRP Conjugate working solution (Elabscience, China), the HRP Conjugate (Elabscience, China) was diluted by a factor of 100 using the HRP conjugate Diluent (Elabscience, China). To each well, 100  $\mu$ L of sample was added and incubated (DNP-9082 Laboratory Incubator) at 37°C for 90 minutes. The liquid was removed from the wells, 100  $\mu$ L of Biotinylated Detection antibody (Elabscience, China) was added, and incubated at 37°C for 60 minutes. The solution was aspirated, and wells were washed thrice. 100  $\mu$ L of HRP Conjugate (Elabscience, China) was added to the wells, incubated at 37°C for 30 min, removed, and washed five times. 90  $\mu$ L of Substrate Reagent (Elabscience, China) was added to the wells, incubated at 37°C for 15 min, and 50  $\mu$ L of Stop Solution (Elabscience, China) was added. At 450 nm wavelength, the optical density of the solution was read immediately with the GF-N3000 Microplate reader, (England) and the results calculated.

The CD3 kit (Bioassay Technology Laboratory, China) was removed from the refrigerator and kept at room temperature for 20 minutes before use. To produce 640 ng/ml standard stock solutions, 120  $\mu$ L of Standard diluent (Bioassay Technology Laboratory, China) was added to 120  $\mu$ L of standard (1280 ng/ml) (Bioassay Technology Laboratory, China). The solution was allowed to stand for

15 minutes before it was serially diluted. 320, 160, 80 and 40 ng/ml solutions were produced. Also, 20 ml of wash buffer concentrate (Bioassay Technology Laboratory, China) was added to 480 ml of wash buffer (Bioassay Technology Laboratory, China). 50  $\mu$ L of standard solution (Bioassay Technology Laboratory, China) was added into the standard well. 10  $\mu$ L anti-CD3 antibodies (Bioassay Technology Laboratory, China) were added to 40  $\mu$ L of sample in the sample wells. To both sample and standard wells, 50  $\mu$ L of streptavidin HRP (Bioassay Technology Laboratory, China) was added. The plate was agitated for thorough mixing, covered, and incubated for 60 minutes at 37°C. With the buffer solution, the plate was washed five times and blotted with paper towel. To each well, 50  $\mu$ L of Substrate Solution A (Bioassay Technology Laboratory, China) and 50  $\mu$ L of Substrate Solution B (Bioassay Technology Laboratory, China) were added. The plate was incubated for 10 min at 37°C. 50  $\mu$ L of Stop solution (Bioassay Technology Laboratory, China) was added to each well. The optical density read immediately using a microplate reader set at 450 nm wavelength.

### 2.5. Statistical Analyses

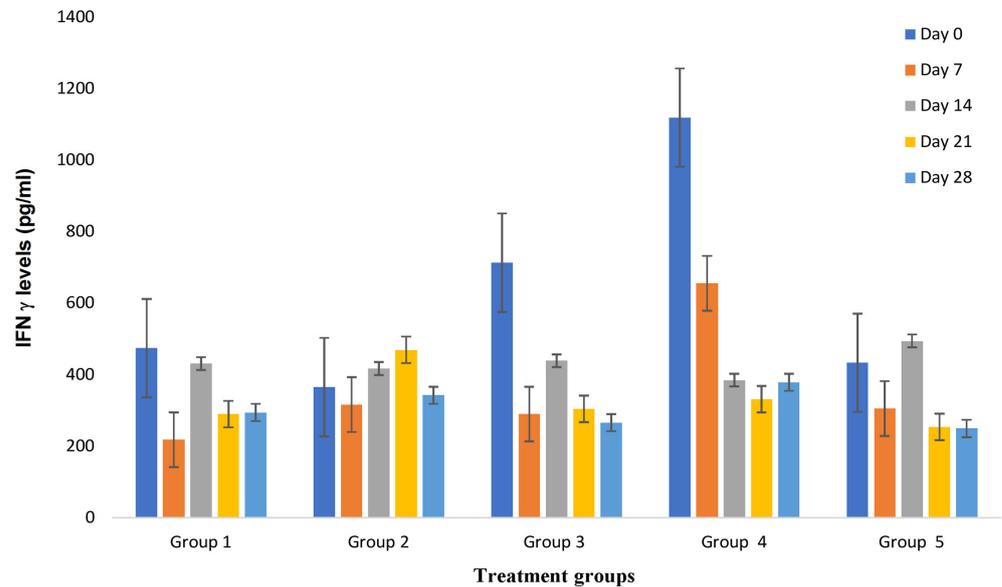
Data collected were analyzed using SPSS version 20 and subjected to one-way Analysis of Variance (ANOVA) in a completely randomized design. Significant means were separated. Using Duncan multiple range Test was and a P value less than 0.05 was considered significant.

## 3. Results

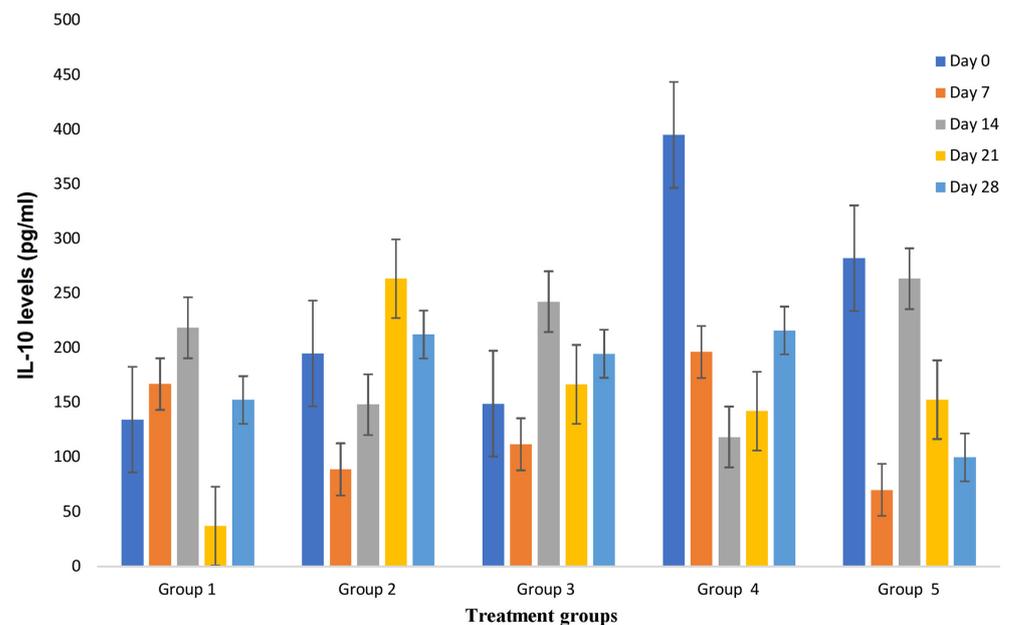
**IFN $\gamma$ :** With respect to the effect of dosage of AEGL, there were significant differences ( $P < 0.05$ ) in IFN  $\gamma$  levels amongst the treatment groups (**Figure 1**). The IFN- $\gamma$  levels (574.25 pg/ml) in rabbits in Group 4 (administered 400 mg/kg AEGL) were higher ( $P < 0.05$ ) than those of the control and other treatment groups. The effect of time was significant ( $P < 0.05$ ) on IFN- $\gamma$  levels declining from 621.20 pg/ml (baseline) to 306.56 pg/ml (day 28).

The trend as seen in Group 1 reveals an undulating trend as IFN  $\gamma$  levels decreased ( $P < 0.05$ ) from Day 0 to Day 7 in all treatment groups (1 - 5), increased from day 7 to day 14 (Groups 1, 2, 3 and 5), decreased from Day 14 to 21 (Groups 1, 3, 4 and 5) and an increase from day 21 to day 28 (Groups 1, 4 and 5). **Figure 1** also reveals high baseline values of IFN  $\gamma$  for Group 3 (713.3 pg/ml) and Group 4 (1119 pg/ml). Group 4 had the highest ( $P < 0.05$ ) value compared to other treatment groups. Put together, the results suggest IFN $\gamma$  levels reduced significantly ( $P < 0.05$ ) from Day 0 to Day7 across all the groups.

**IL 10:** The effect of AEGL dosage on IL-10 was not significant ( $P > 0.05$ ) as shown in **Figure 2**. However, levels seen in groups 3, 4 and 5 (administered 200, 400 and 600 mg/kg AEGL, respectively) were higher than those of the control groups (Group 1 and 2). The effect of time was significant ( $P < 0.05$ ) on IL-10 levels declining from 235.15 pg/ml (baseline) to 126.90 pg/ml (day 7) and 152.39 pg/ml (day 21).



**Figure 1.** Effect of the AEGL concentration and time on the expression of IFN- $\gamma$ .



**Figure 2.** Effect of the AEGL concentration and time on the expression of IL-10.

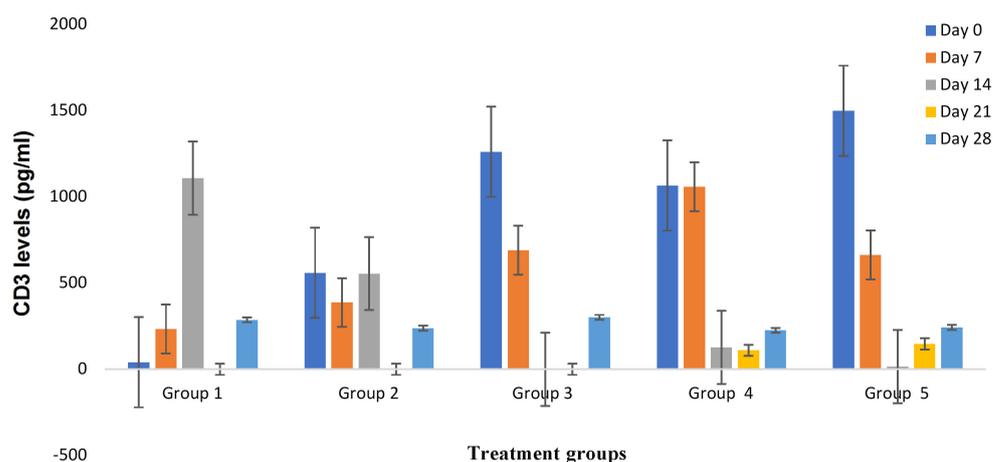
The trend seen in **Figure 2** reveals that Group 4 maintains a steady decline from baseline levels to Day 14. However, undulating trends are observed as cytokine levels decrease from Day 0 to Day 7 (groups 2, 3, 4 & 5) increase from Day 7 to Day 14 (groups 1, 2, 3 & 5), decrease from day 14 to 21 (Group 1, 3 and 5) and increase from day 21 to 28 (groups 1, 3 and 4). Group 1 increases from baseline value, peaked at Day 14 and declines. **Figure 2** also reveals that highest ( $P < 0.05$ ) baseline values of 395 pg/ml were recorded in Group 4. A significant reduction in IL10 activity over a 14-day period suggests that the greatest IL10-inhibitory activity occurred at 400 mg/kg of AEGL when administered for 14

days. At 400 mg/kg, AEGL showed a stronger IL10-inhibitory activity than 0.33 ml/kg of Multivitamins (Group 2) whose activity only lasted for 7 days.

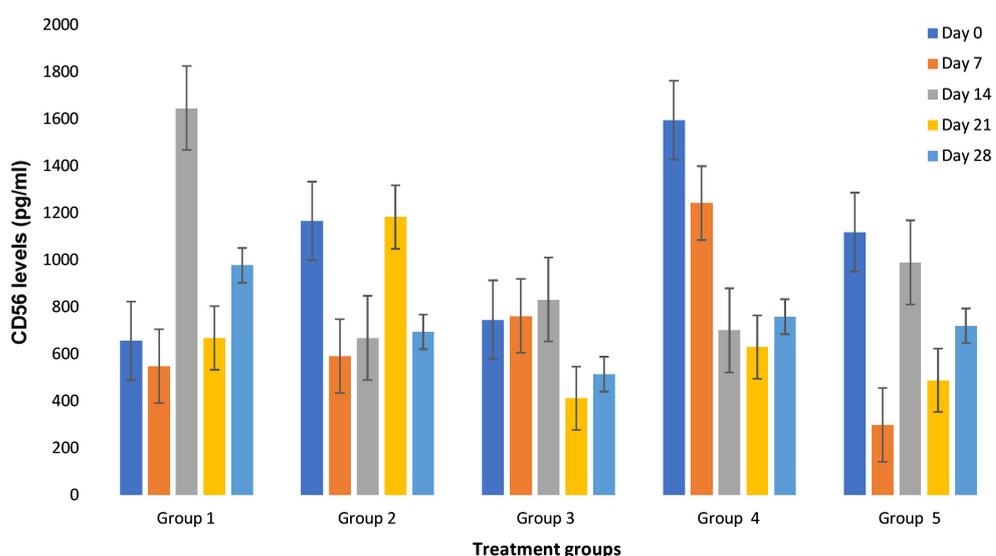
**CD3:** The effect of AEGL dosage on CD3 was not significant ( $P > 0.05$ ) as seen in **Figure 3**. The effect of time was significant ( $P < 0.05$ ) on CD3 levels declining from 884.60 pg/ml (baseline) to 51.16 pg/ml (day 21)

The trend in **Figure 3** reveals that the baseline values for CD3 in Group 3 (1259.8 pg/ml), Group 4 (1065 pg/ml) and Group 5 (1248.5 pg/ml) were comparably higher ( $P < 0.01$ ) than those of other treatment groups. A decline ( $P < 0.01$ ) in CD3 levels was seen from day 0 to 7 in groups 2, 3, 4 and 5 until Day 14. CD3 levels in groups 2 and 3 remained undetectable from days 14 to 21. All groups showed recovery from day 21. Group 1 showed an opposite trend to all the other groups till day 21.

**CD56:** The effect of AEGL dosage on CD56 was not significant ( $P > 0.05$ ) as shown in **Figure 4**. The effect of time was significant ( $P < 0.05$ ) on CD56 levels



**Figure 3.** Effect of the AEGL concentration and time on the expression of CD3.



**Figure 4.** Effect of the AEGL concentration and time on the expression of CD56.

declining from 1056.87 pg/ml (baseline) to 676.84 pg/ml (day 21). Is the archetypal immunophenotypic marker of natural killer cells and triggers effector functions such as cytotoxicity and T helper 1 cytokine production (Van Acker *et al.*, 2017). **Figure 4** shows that the highest CD56-inhibition is at 400 mg/kg.

Trend seen showed a marked drop ( $P < 0.01$ ) in CD56 levels from day 0 to 7 in all treatment groups. This was successively followed by an increase in cytokine levels ( $P < 0.01$ ) in groups 1, 2, 3 and 4 from days 7 to 14. Days 14 - 21 was characterized by a decline in CD56 levels while days 21 to 28 was seen in groups 1, 3, 4 and 5.

#### 4. Discussion

**IFN $\gamma$ :** Certain bioactive compounds contained in *Gongronema latifolium* have anti-inflammatory and antioxidant activities [11]. Like several other plant extracts [14] [15], AEGL can be used for the treatment of inflammation as they have been proven to decrease IFN- $\gamma$  activity. The down regulation of the expression of TNF- $\alpha$ /IFN- $\gamma$  through the blockade of several pathways is associated with the inhibition of ERK1/2 phosphorylation [13] [16].

It has been shown that many plants with anti-inflammatory effect could also some antibacterial properties [17] [18]. The roughages taken by Group1, Tridax pubescens and the multivitamins taken by Group 2 could also reduce IFN  $\gamma$  levels by their anti-inflammatory activity [19]. An upward significant ( $P < 0.05$ ) trend in seen Group 3 from Days 7 and 14 is suggestive of an NKC-derived IFN  $\gamma$  production. Like bitter gourd (*Momordica charantia*) juice and the 70% hydro-methanolic extract of *Dioscorea alata*, AEGL may also show pro-inflammatory activity by increasing IFN  $\gamma$  production [20]. IFN $\gamma$  produced by NKC has been shown to have cytotoxic activity [21].

Over the 28-day period, IFN $\gamma$  production is most significantly ( $P < 0.05$ ) altered in Group 4 (400 mg/kg of AEGL). IFN $\gamma$  levels reduced from 1119 pg/ml to 332 pg/ml in a 21-day period showing the greatest inhibitory activity. This suggests a maximum effective dose of 400 mg/kg of AEGL administered at 21 days. High baseline values for Group 3 and 4 might be suggestive of a pre-existing inflammatory state where macrophages are activated which in turn trigger the production of Th1-derived IFN $\gamma$  [22].

**IL-10:** Results suggests that AEGL is a natural inhibitor of IL10. In a similar fashion, Rituximab, a chimeric mouse antihuman CD20 antibody, inhibits IL10 resulting in the down regulation of bcl-2 and sensitization of B-cell non-Hodgkin's lymphoma to apoptosis caused by chemotherapeutic drugs [23]. IL10 is an immunosuppressive cytokine which directly affect NKC count. Elevated IL10 levels in a tumor microenvironment, suppresses IL12 which is the main stimulator of NKC production and it also down-regulates the expression TNF, and IFN- $\gamma$  [23]. On the contrary, with low IL10 levels, IL12 is increased and this increases NKC cytotoxicity [24].

**CD3:** CD3 is an important and highly specific immunohistochemical marker

for all subtypes of T-cells including immature T cells like pro-thymocytes and thymocytes. It is also marker for T cell lymphomas (differentiating them from B cell lymphomas) and leukemia [25] [26]. CD3 binds with subunits of TCR (alpha/beta ligand binding subunits) to form a complex that triggers signal transduction. Ligand binding with this complex induces conformational change of CD3 and recruits Nck adaptor protein [27]. The CD3 result shows the treated control and other treated groups caused the inhibition of CD3 T-cell proliferation. This might be due to the presence of common compounds such as Vitamin D. 1, 25-dihydroxyvitamin D3 which has been shown to inhibit the proliferation of T lymphocytes [27] [28].

**CD56:** is neural cell adhesion molecule. It is a typical example of phenotypic marker of natural killer cells and so cytotoxicity. The 400 mg/kg AEGL had the most significant up-regulatory effects on the marker ( $P < 0.05$ ). The does may serve to for the detection of cytotoxicity.

Though known for its remarkable antioxidant and NKC stimulatory properties, the administration of 0.33 ml of multivitamins per kg live weight did not show enough potency as compared with 400 mg/kg of AEGL. High baseline values in groups 3 and 4 could be suggestive of an inflammatory process. Group 3 showed the highest inhibition of T cell activation while Group 4 showed the highest NK cell activity. However, it is important to note that the results and the interpretations may have been negatively affected by several factors which include the complex cytokine network interactions, nature of cytokines, genetic differences, experimental methodology and sample related issues [29] [30].

#### **Established Facts:**

- Gongronema latifolium is a perennial edible plant with soft and pliable stem
- Medicinal plants, including Gongronema latifolium, have anti-inflammatory activity
- Medicinal plants have been used from the ancient times for human health benefits

#### **Highlights of the findings and novelties:**

- At 400 mg/kg for 21 days in rabbits, AEGL showed very strong anti-inflammatory activity against all the cytokines and cell surface markers
- The molecular mechanism is through the expression of IFN $\gamma$  and IL-10 cytokines via the expression of CD3 and CD56 cellular sources
- Administration of 0.33 ml of multivitamins per kg live weight did not show enough potency as compared with 400 mg/kg of AEGL

## **5. Conclusion**

In general, the production of all the cytokines was affected in time while IFN- $\gamma$  levels increased in a dose-dependent manner following the administration of AEGL. IFN $\gamma$  showed significant anti-inflammatory and pro-inflammatory activities because of their pleiotropic nature. The rabbits that received 400 mg/kg for all the cytokines exhibited significant activity which is suggestive of the maximum effective dose for AEGL (21 days for IFN $\gamma$  and 14 days for IL10).

## Acknowledgements

Authors wish to acknowledge the management of Nigerian Institute of Pharmaceutical Research and Development (NIPRD) for permission to use the institutions facility to run the studies.

## Conflicts of Interest

Authors declare, there is no conflict of interest.

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## List of Abbreviations

Natural Killer Cell (NKC)

Aqueous extract of *Gongronema latifolium* (AEGL)

Enzyme-linked immunosorbent assay (ELISA)

National Institute for Pharmaceutical Research and Development (NIPRD)

Horseradish peroxidase (HRP)

Standard error in the mean (SEM)

Analysis of Variance (ANOVA)