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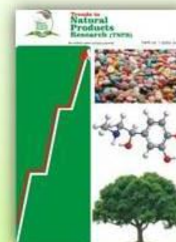

# Immunomodulatory Properties of *Combretum calobotrys* Engl. and Diels (Combretaceae) Leaf Extract

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**Keywords:** *Combretum calobotrys*, antibody, immunomodulation, hypersensitivity, leucocyte mobilization

**Abstract:** The leaves, stem barks and roots of *Combretum calobotrys* Engl. and Diels (Combretaceae) have been used over the years in South-Eastern Nigeria for anti-inflammatory, anti-nociceptive, anti-microbial and immune-stimulating activities. This study evaluated the immunomodulatory properties of *C. calobotrys* leaf extract (CCE) in rodents using *in vivo* leucocyte mobilization test, humoral antibody synthesis (HAS) and delayed-type hypersensitivity (DTH) reaction. Acute toxicity and lethality tests as well as phytochemical screening of CCE were also performed. The CCE up to 5000 mg/kg administered orally showed no sign of toxicity after 24 hr observation period. The CCE increased carrageenan-induced mobilization of total leucocytes into the peritoneal cavity. The CCE (250 mg/kg) caused elevation of both primary (4.8±1.46) and secondary (4.7±0.37) sheep red blood cells specific antibody titres, compared to the control (2.40±0.98 for primary and 4.60±0.40 for secondary). The CCE also stimulated delayed-type hypersensitivity responses. The CCE gave positive reactions for alkaloids, saponins, flavonoids, tannins, terpenoids, steroids, resins, proteins, carbohydrates and reducing sugars. The results demonstrated that *C. calobotrys* leaf possesses immunomodulatory properties. Lethality tests as well as phytochemical screening of CCE were also performed. The CCE up to 5000 mg/kg administered orally showed no sign of toxicity after 24 hr

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Page No.: 34-41
Volume: X, Issue 1, 2020
Trends in Natural Products Research
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Observation period. The CCE increased carrageenan-induced mobilization of total leucocytes into the peritoneal cavity. The CCE (250 mg/kg) caused elevation of both primary (4.8±1.46) and secondary (4.7±0.37) sheep red blood cells specific antibody titres, compared to the control (2.40±0.98 for primary and 4.60±0.40 for secondary). The CCE also stimulated delayed-type hypersensitivity responses. The CCE gave positive reactions for alkaloids, saponins, flavonoids, tannins, terpenoids, steroids, resins, proteins, carbohydrates and reducing sugars. The results demonstrated that *C. calobotrys* leaf possesses immunomodulatory properties.

## INTRODUCTION

The immune system plays a vital role in the management of infections (Paul, 2013). Modulation of the immune response in order to mitigate diseases is a research area with growing, evolving and robust opportunities. Immunomodulation, which could be either immuno-stimulation (potentiation of immune response) or immuno-suppression (weakening of immune response), involves regulating the immune response using agents from plants, animals or synthetic origin (Rajeev, 2019). The outbreaks of pandemics of viral origin in recent years have posed a serious crisis to the human race. More specifically, the recent COVID-19 pandemic weakens the immune system. Many agents used to improve immune function are synthetic agents and as such, they are associated with serious side effects and the poor may not be able to afford them. Consequently, natural products, are being employed in boosting body's immunity. Many plants have been investigated for their immunomodulatory properties (Bhat *et al.,* 2015, Khusnawati *et al.,* 2015, Mansouri *et al.,* 2015, Ranganathan and Selvasubramanian, 2015, Das *et al.,* 2016, Magrone *et al.,* 2016, Singh *et al.,* 2016, Munawaroh *et al.,* 2018, Aji, 2019). One of the plants with putative immunomodulatory activity is *Combretum calobotrys* Engl. and Diels (Combretaceae), a flowering plant that is well distributed in the tropics of America, Asia, and Africa (Lu and Phillips, 2009). The morphological characteristics of the plant have been described in details (Lu and Phillips, 2009). The leaf and bark of *Combretum* species are used in many parts of Africa to manage abdominal discomfort, body pains, respiratory disorders, fever, worm infestations, infertility, leprosy, syphilis, inflammation, malarial and other protozoal infections (Ojewole, 2008; Denis *et al.,* 2013), as well as some other disease states where the immune system is involved. Although the anti-inflammatory and antimicrobial properties of *C. calobotrys* have been documented (Ezike *et al*., 2011, 2013), a knowledge gap in literature is evident as scientific reports on immunomodulatory activity of *C. calobotrys* appears to be unavailable. With the challenges arising from the COVID-19 pandemic and also bearing in mind the side effects and/or adverse effects associated with corticosteroids prolonged use in improving immune function, there is an urgent need to identify a new cost-effective and safer approach to improve the immune function. In view of this and to also justify the ethnomedicinal use of *C. calobotrys* in improving immune function, the objective of this study was to evaluate the immunomodulatory properties of *C. calobotrys* leaves.

## MATERIALS AND METHODS

### Collection of plant material and preparation of extract

Fresh leaves of *C. calobotrys* were collected from Udi, Enugu State, Nigeria between November and December. The plant was identified and authenticated by Mr Alfred O. Ozioko of the International Centre for Ethnomedicine and Drug Development (InterCEDD) Nsukka, Enugu State (voucher number: Intercedd/26982). The leaves were washed in clean water, cut into small pieces, dried under shade for 3 days and pulverized to coarse powder using a mechanical grinder. The powdered leaf (3 kg) was extracted by cold maceration in a 1:1 mixture of methanol and dichloromethane for 48 hr, concentrated in a rotor evaporator under reduced pressure, and completely dried over a water bath at 60ºC to yield 250 g of methanol-dichloromethane extract (CCE; 8.33 % w/w).

### Animals

Adult outbred strain of albino mice (20-25 g), and Sprague Dawley rats (100-120 g) of either sex bred in the Laboratory Animal Facility of the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka were used. A male sheep was procured from the herd of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka. All animal experiments were in compliance with internationally accepted principles for laboratory animal use and care as found in the National Institute of Health Guide for Care and Use of Laboratory Animals (NIH publication #85-23, revised in 1985).

### Determination of acute toxicity and $LD_{50}$

The acute toxicity and lethality ($LD_{50}$) of CCE was determined in line with the method described by Lorke (1983). Briefly, nine mice randomly divided into three groups (n = 3) were orally administered 10, 100, and 1000 mg/kg of CCE, respectively and

observed for 24 hr for signs of toxicity or death. Since no death was recorded, 1,600, 2,900 and 5,000 mg/kg of CCE were administered, respectively to a fresh batch of animals (n = 1) and they were also observed for 24 hr for signs of toxicity or death. The $LD_{50}$ was calculated as the geometric mean of the highest non-lethal dose and the least lethal dose.

**Phytochemical screening**

Phytochemical analysis of CCE was carried out as outlined by Trease and Evans (1983).

**Antigen (sheep red blood cells) preparation**

Fresh sheep blood (10 ml) was aseptically taken from the jugular vein of a healthy male sheep and transferred to an EDTA bottle. Sheep red blood cells (SRBCs) were washed thrice in copious quantity of pyrogen-free sterile normal saline by centrifugation at 3000 rpm for 10 min on each occasion. The SRBCs was adjusted to a concentration of $10^9$ cells/ml with sterile normal saline and used for immunisation and challenge.

***In vivo* leucocyte mobilization test**

The effects of CCE on *in vivo* leukocyte mobilization was evaluated using the method of Ribeiro *et al.,* (1991). Rats were randomly divided into 5 groups of five animals each (n = 5). Treatment was as follows - groups I, 2 and 3 received 100, 250 and 500 mg/kg of CCE respectively while groups 4 and 5 received levamisole 2.5 mg/kg (positive control) and normal saline (2.5 ml/kg, negative control) respectively via the oral route. One hour after administration of the extract, each rat received 0.5 ml of 1 % w/v carrageenan suspension in normal saline via the intraperitoneal route. Four hours later, the rats were sacrificed and the peritoneal cavity washed with 5 ml of a 5 % solution of EDTA in phosphate buffered saline (PBS). Total and differential leucocyte counts (TLC and DLC) of the perfusates were determined.

**Humoral antibody synthesis**

The effects of CCE on humoral antibody synthesis (HAS) was evaluated as described by Nelson and Mildenhall, (1967) with some modifications. Rats were randomly divided into 6 groups of five animals each (n = 5). Treatment was as follows - groups 1, 2 and 3 received 100, 250 and 500 mg/kg of CCE respectively while group 4 received levamisole (2.5 mg/kg, positive control) and group 5 received normal saline (2.5 ml/kg, negative control) via the oral route. The CCE was administered 3 days prior to sensitization and continued daily for 5 days after the challenge. On day zero, 0.1 ml of $10^9$ cell/ml SRBC was given though the intraperitoneal (IP) route to all the groups for sensitization. The animals were challenged on day 5 by similar IP injection of the same amount of SRBC. Primary antibody titre was determined on day 5 (before the challenge) and secondary titre on day 10. Blood samples were obtained by retro-orbital puncture into test tubes and allowed to clot. A 25 µl serum for each sample was obtained after centrifugation and serially diluted two-fold in 96-U well microtitre plates using pyrogen-free normal saline. The last well on each row contained sterile normal saline as control. The diluted sera were challenged with 25 µl of 1 % (v/v) SRBC in the plates and then incubated at 37°C for 1 hr. The highest dilution giving rise to visible hemagglutination was taken as antibody titre. Antibody titres were expressed in graded manner, the minimum dilution (1/2) being ranked as 1 (calculated as $\log_2$ of the dilution factor).

**Delayed type hypersensitivity reaction**

The effects of CCE on Delayed type hypersensitivity (DTH) reaction was evaluated as described by Shinde *et al*., (1999) with some modifications. Briefly, 30 rats were randomly divided into 6 groups consisting of 5 animals each (n = 5). Delayed type hypersensitivity was induced in mice using SRBC. Treatment was as follows; groups 1, 2 and 3 received 100, 250 and 500 mg/kg of the extract respectively while group 4 received levamisole (2.5 mg/kg, positive control) and group 5 received normal saline (2.5 ml/kg, negative control) via the oral route. The animals were treated with the extract 3 days prior to sensitization and continued till the challenge. On day zero, 1 hr after extract administration, the rats were sensitized by injecting 0.02 ml of $10^9$ cells/ml of SRBC into the right hind paw subcutaneously (SC). The animals were challenged on day 5 by SC injection of the same amount of antigen into the left hind paw. The edema produced by antigenic challenge in the left hind paw was measured as the difference in the paw thickness before and 24 hr after the challenge. Percentage inhibition was calculated using the formula;
Inhibition (%) = (1- mean of treatment/mean of control) x 100

**Statistical analysis**

The results were expressed as Mean ± Standard Error of Mean and analyzed using GraphPad Prism version 7.0. Differences between means of treated and control groups were evaluated further using LSD Post hoc test and considered significant at $p < 0.05$.

## RESULTS

**Acute toxicity and $LD_{50}$ of *C. calobotrys* leaf extract**

The CCE was found to be relatively safe ($LD_{50}$ >5000 mg/kg) since no death was recorded at 5000 mg/kg after 24 hr observation period.

**Phytochemical analysis**

The CCE gave positive reactions for alkaloids, saponins, resins, flavonoids, tannins, proteins, steroids, carbohydrates, reducing sugars and terpenoids (Table 1).

**Effect of the extract on leucocyte mobilization**

The CCE (250 and 500 mg/kg) caused an increase in carrageenan-induced total leucocyte mobilization into the peritoneal fluid, compared to the control. While CCE (100 mg/kg) and levamisole caused slight decrease in TLC. Also, CCE decreased neutrophil migration in a dose-related manner, while migration of lymphocytes was increased. Generally, CCE mobilized the lymphocytes more than neutrophils (Table 2).

**Effect of the extract on humoral antibody synthesis**

The CCE (250 mg/kg) caused elevations of primary and secondary SRBC specific antibody titres, respectively compared to the control (Figure 1).

**Effect of the extract on delayed type hypersensitivity reaction**

The CCE stimulated delayed type hypersensitivity reaction in a dose-related manner, with 250 and 500 mg/kg eliciting significant ($p < 0.05$) response. The immunostimulation produced by 250 and 500 mg/kg were greater than that of the standard immunostimulant, levamisole (Figure 2).

**Table 1: Phytoconstituents of *C. calobotrys* leaf extract (CCE)**

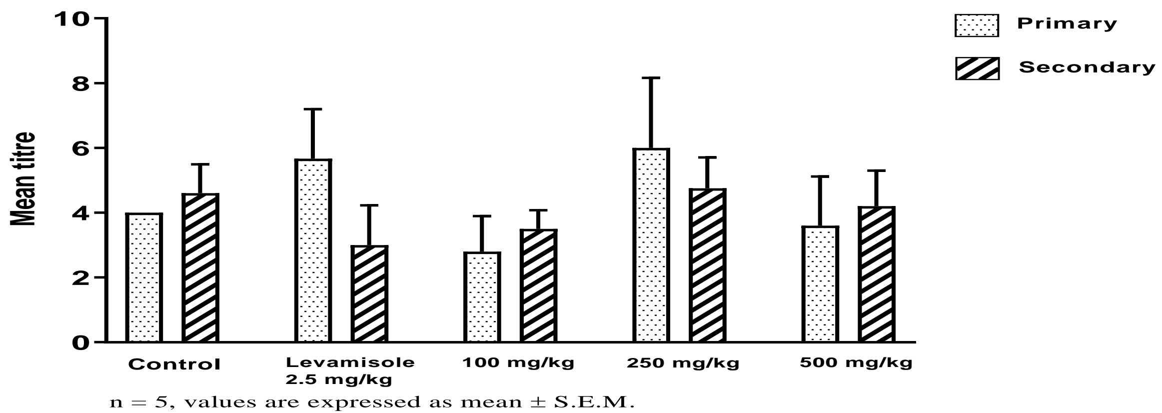
| Phytoconstituent | Relative presence |
|---|---|
| Alkaloids | +++ |
| Saponins | + |
| Flavonoids | +++ |
| Tannins | + |
| Terpenoids | + |
| Steroids | + |
| Resins | ++ |
| Glycosides | + |
| Carbohydrates | +++ |
| Reducing sugars | + |
| Proteins | ++ |
| Fats and oil | + |

+ = mildly present, ++ = moderately present, +++ = highly present, ++++ = abundantly present

**Table 2: Effect of *C. calobotrys* leaf extract on leukocyte mobilization**

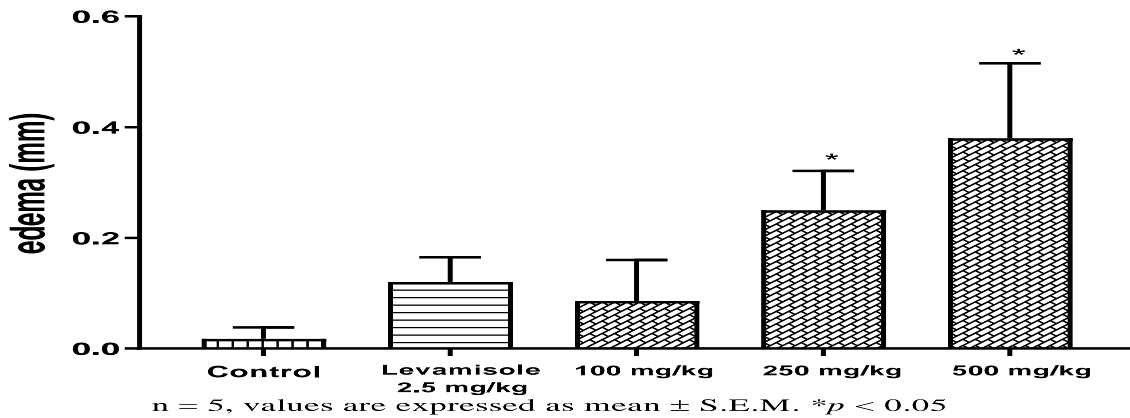
| Treatment | Dose ( mg/kg) | TLC | DLC | |
|---|---|---|---|---|
| | | | Neutrophils | Lymphocytes |
| CCE | 100 | 746±112 | 23.6±4.5 | 76.4±4.5 |
| | 250 | 1100±182 | 22.6±2.7 | 77.4±2.7 |
| | 500 | 1350±387 | 19.8±4.0 | 80.2±4.0 |
| Lev | 2.5 | 990±95 | 28.0±5.0 | 72.0±5.0 |
| Control | - | 996±273 | 24.0±1.7 | 73.4±2.6 |

n = 5, CCE = crude extract, lev = levamisole, TLC = total leukocyte count, DLC = differential leukocyte count




n = 5, values are expressed as mean ± S.E.M.

**Figure 1:** Effect of CCE on humoral antibody synthesis




n = 5, values are expressed as mean ± S.E.M. $*p < 0.05$

**Figure 2:** Effect of *C. calobotrys* leaf extract on delayed type hypersensitivity reaction in rats

## DISCUSSION

The *C. calobotrys* leaf extract stimulated immune response in the various experimental models employed in this study.

The extract increased leucocyte mobilization into the peritoneal fluid when compared to the control in a dose dependent manner. The lymphocytes were highly increased depicting immunostimulatory property. Circulating blood leucocytes are required to migrate to sites of tissue injury and infection with the principal aim of eliminating the primary inflammatory trigger and contributing to tissue repair (Ley *et al*., 2007). In addition, antigens, largely through activation of resident memory T cells, can trigger recruitment of leucocytes via secretion of various primary inflammatory cytokines (Ley *et al*., 2007). Tissue sentinel cells, including mast cells, macrophages, and dendritic cells, play a key role in detection of such danger signals and can release a wide range of pro-inflammatory mediators to promote leucocyte recruitment (Ley *et al*., 2007). The primary step in leucocyte migration is the establishment of weak and transient adhesive interactions between leucocytes and endothelial cells of post-capillary veinular walls in close vicinity to inflamed tissues (Ley *et al*., 2007). This facilitates *in situ,* stimulation of leucocytes by endothelial presented chemo-attractants displayed on the luminal side of blood vessels, propagating firm leucocyte arrest, adhesion strengthening, crawling, and subsequently migration of cells out of the blood vasculature (Ley *et al*., 2007). These cues govern the site and route of leucocyte migration along and through the endothelial cell barrier, determining a potential need for chemotactic crawling on the apical aspect of the endothelium to seek permissive sites and/or additional exit cues (Stark *et al*., 2013).

Most notably, transmigrated leucocytes exhibit altered phenotype, enhanced survival, and increased effector functions, such as greater ability to kill and clear invading pathogens and tumor cells (Stark *et al*., 2013). Consequently, breaching of veinular walls not only provides a regulated process for facilitating leucocyte migration into inflamed tissues but also acts as a key process though which tissue-infiltrated leucocytes are primed for delivering an effective immune response (Stark *et al*., 2013). The importance of this action is that the extract may likely quicken cellular response to inflammation and clearly indicates its immunostimulating properties.

The extract at different doses, elicited varying effects on the primary and secondary antibody titres in a non-dose dependent manner. This effect may also be attributed, albeit partly, to the increase in lymphocytes observed in the *in vivo* leucocyte mobilization test since antibody molecules, a product of B lymphocytes and plasma cells, are central to humoral immune responses (Michael, 2013). Humoral mediated immunity involves the production of specific antibodies (immunoglobulins) by bursa equivalent lymphocytes or plasma cells following sensitization to specific antigen (Edward and Sebastian, 2013). Antibody synthesis requires the cooperation of at least three major cell types, the macrophages, B lymphocytes, and T lymphocytes while antibodies such as immunoglobulin G and immunoglobulin M are principally involved in the complement activation, opsonization and neutralization of toxin (Paul, 2013). A potent immunostimulant should be able to stimulate sufficient production of antibodies to help fight infection (Paul, 2013). This property will enhance humoral immune protection of the animal which is mediated though opsonization, direct neutralization of antigen, agglutination of antigen and activation of complement system to cause lysis and death of the antigenic cells (Paul, 2013). This result suggests that the extract may be a good candidate for stimulating immune function.

The extract significantly stimulated delayed type hypersensitivity reaction in a dose dependent manner. The DTH reaction is mediated by interferon-gamma producing $CD4^+$ or $CD8^+$ T-cells (Allen, 2013, Sachdeva *et al*., 2014). Also, DTH reaction is known to be initiated by reaction between antigen-specific T-cells and the antigen which results in the release of lymphokines that affect a variety of cell types especially macrophages (Paul, 2013, Allen, 2013). Several lines of evidence suggest that DTH reaction is important in host defense against parasites and can live and proliferate intracellularly (Sachdeva *et al*., 2014). When challenged by the antigen, T-lymphocytes are converted to lymphoblasts and secrete a variety of molecules including pro-inflammatory lymphokines, attracting more scavenger cells to the site of reaction (Paul, 2013), and the infiltrating cells are probably immobilized to promote the defensive (inflammatory) reaction (Abbas *et al.,* 2016). An increase in delayed type hypersensitivity response exhibited by the extract indicates that it may possess a stimulatory effect on lymphocytes.

Phytochemical screening revealed the presence of some phytoconstituents which were in accordance with the results reported by Ezike *et al*., (2013). Studies have shown that different types of flavonoids may stimulate human peripheral blood leucocyte proliferation and flavonoids have also been reported to cause increase in the helper T-cells, interleukin II, interferon and macrophages; hence they are useful in several diseases of immune dysfunction (Chiang *et al*., 2003). Though no specific phytoconstituent could be linked to the activity of *C. calobotrys* leaf extract at this stage of the work, the presence of these phytoconstituents could be responsible for the immunomodulatory activity of the extract.

## CONCLUSION

The results of this study demonstrated that *C. calobotrys* leaf extract may possess immunomodulatory properties. This probably justifies the traditional use of *C. calobotrys* plant in improving immune function.

## CONFLICT OF INTEREST

The authors have not declared any conflict of interest.

## FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## CONTRIBUTION OF EACH AUTHOR

**Ezike, Adaobi Chioma**; conceptualised and designed the research; conducted the experiments and edited the manuscript
**Ibe, Jane**; conducted the experiments and data analysis
**Isiogugu, Ogechukwu Nnanyelugo;** participated in data analysis and prepared the manuscript

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