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Research Article

Ethyl acetate fraction of *Diaphananthe bidens* leaf extract showed immunomodulatory activity in experimental mice models

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Abstract

The ethyl acetate fraction of *Diaphananthe bidens* leaves was tested in mice for immunomodulatory effects. Specific and non-specific responses to humoral and cellular components of the immune system were evaluated. The mice were observed for 28 days in five groups. Control group 1 received 10 mg/kg Tween 20, while positive control group 2 received levamisole (5.75 mg/kg). Groups 3-5 received *D. bidens* ethyl acetate fractions at 93, 186, and 372 mg/kg. On day 25, sensitized mice were challenged with sheep red blood cells. An immunostimulatory dose-response assay, haemagglutination titre, and ovalbumin antibody response were used to assess humoral antibody response on day 29. Each group's delayed-type hypersensitivity (DTH) response was measured using a digital Vernier calliper. Blood samples were taken to measure the phagocytic index. The sheep red blood cells (SRBC) antigenic stimulation induced anti-SRBC immunologic stimulation. Immunostimulation increased dose-dependently ($P<0.05$) with the ethyl acetate fraction, peaking at 372 mg/kg compared to the reference drug ($P<0.05$). Ovalbumin-specific total IgG, IgG_1, and IgG_{2a} against ovalbumin antigens are also induced by the ethyl acetate fraction of *D. bidens*. The fraction doses had significant ($P<0.05$) primary, secondary, and tertiary responses compared to vehicle control groups on the 7th and 14th day post-boost. Due to its dose-dependent DTH response to ovalbumin, ethyl acetate had significant ($P<0.05$) dose-related effects. The dose-related phagocytosis activities of ethyl acetate were significantly ($P<0.05$) higher than the vehicle. As it is used for a variety of medicinal purposes, *D. bidens'* immunomodulatory effects in mice support its folkloric uses.

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1. Introduction

Secondary metabolites derived from plants and herbs have been shown to potentiate or suppress the immune system [1]. Some medicinal herbs have been reported to possess anti-inflammatory, anti-oxidant and anti-cancer effects by modulating immune functions [2-4]. The immune system is composed of three tiers of protection: the physical barrier, the innate immune system, and the adaptive immune system, whose function is to balance the humoral and cellular responses of the adaptive immune system [5]. Immunomodulation is essential in managing several illnesses, such as autoimmune disorders, cancer, and

infectious diseases. Singh et al. [2] reported immunomodulatory effects of *Ocimum sanctum* by regulating the IL-2 production and its mRNA expression using rat splenocytes. It was also found that *Withania somnifera* promotes immunity [6]. Other reported plant extracts with immunomodulatory are *Astragalus membranaceus* , *Echinacea purpurea* and *Allium sativum* [7] among others.

Diaphananthe bidens, a tropical plant species belonging to the *Orchidaceae* family has been utilized in traditional medicine to treat various chronic diseases like rheumatoid arthritis, and other inflammatory conditions [8]. Some of the reported pharmacological activities include anti-inflammatory, antibacterial, and antioxidant [9-11], and thus, possess hepatoprotective and anti-hyperglycemic properties. Nonetheless, the immunomodulatory potential of *Diaphananthe bidens* remains predominantly unexplored. In this study, we report its Immunomodulatory effects in experimental mice models.

2. Materials and methods

2.1. Plant collection

The leaves of *Diaphananthe bidens* were collected in June 2023 from Nsukka, Enugu State, Nigeria. Mr. Alfred Ozioko, a Taxonomist from the Bioresources Development and Conservation Project (BDCP) in Nsukka, collected and authenticated the leaves. The voucher specimen was deposited in the herbarium of the Department of Pharmacognosy and Traditional Medicine, located in the Faculty of Pharmaceutical Science at Nnamdi Azikiwe University's Agulu Campus. Leaves weighing 12 kg had a thorough cleaning and were then dried in the shade for 28 days. A dry sample weighing 6.7 kg was pulverized using a mechanical grinder (Gx160 Delmar 5.5 HP, Honda Motor Co. Ltd., Japan) and subsequently utilized for solvent extraction.

2.2. *Animals*

The study utilized adult mice weighing between (20 - 25 g). These animals were sourced from the Animal House of the Department of Pharmacology, Faculty of Pharmaceutical Science, Enugu State University of Science and Technology, located in Enugu State, Nigeria. The animals were provided with pelletized meals from Vital Feeds, a company based in Nigeria, and had unrestricted access to water. The animals were housed in normal cages at the Animal House of the Department of Pharmacology. The animals had a 7-day acclimatization period before being utilized for the investigation. The animal studies were carried out strictly following the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Nnamdi Azikiwe University Animal Research and Ethics Committee under the approval number NAU/AREC/02024/0088.

2.3. Extraction, fractionation and phytochemical analysis

2.3.1. Extraction

A 6 kg quantity of pulverized leaves of *D. bidens* was cold macerated in 30 L of aqueous ethanol (70%) for 72 h with intermittent shaking. The resulting solution was filtered, and the filtrate was pre-concentrated *in vacuo* using a rotary evaporator at 40°C. Subsequently, it was dried to a constant weight using an open water bath at the same 50°C to obtain the ethanol extract [12].

2.3.2. Fractionation (Liquid-liquid Chromatography)

The ethanol extract (100 g) was dissolved in distilled water and subjected to liquid-liquid partition successively with 2.5 L of n-hexane, ethyl acetate, and then butanol using a separating funnel to obtain the n-hexane, ethyl acetate and butanol soluble fractions, respectively. The remaining fraction after the partitioning was taken as the water fraction. The fractions were pre-concentrated using a rotary evaporator at 40^{o}C and dried using a water bath at 50^{o}C. The water fraction was freeze-dried at -50^{o}C using a Telstar LyoQuest freeze dryer.

2.3.3. Phytochemical analysis

The phytochemical analysis of the leaf extract and fractions was carried out using standard methods [13,14].

2.3.4. Total phenolic content by Folin Ciocalteu's assay

The total phenolic content of the extracts and fractions was determined using the method described by Kim et al. [10]. One millilitre of the samples (62.5 µg/mL) was mixed with 0.2 mL of Folin-Ciocalteu's phenol reagent. After 5 minutes, 1 mL of 7.6% Na_2CO_3 solution was added to the mixture, followed by 2 mL of distilled water. The mixture (in duplicate) was incubated at 40°C for 30 minutes, after which the absorbance was read at 760 nm using a UV-VIS spectrophotometer (Model 752, China). The total

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phenolic content was estimated from the calibrated curve by preparing the gallic acid solution and expressed as milligrams of gallic acid equivalent (GAE) per gram of the extracts [15, 16].

2.4. Determination of effective dose (ED_{50}) from immunosuppressed mice

The study was conducted with mice divided into control and treatment groups. Graded doses of the extract and fractions of *D. bidens* were administered to their respective groups of six mice per group. Group 1 served as the vehicle control group and received 10 mL/kg of 5% Tween 20. Groups 2 - 6 were given graded doses (50, 150, 300, 600 and 1200 mg/kg) of the extract. Groups 7 – 11 were graded doses of ethyl acetate fraction. Groups 12 – 16 received graded doses (5, 15, 30, 60 and 120 mg/kg) of the reference standard – levamisole. The same doses used for the extract were also applied for the fractions. Treatments were done orally for 4 weeks (28 days). However, the animals' immune systems were suppressed on the 25th day of the study – two days after antigenic stimulation with 0.2 mL of SRBC – by intraperitoneal injection of 80 mg/kg cyclophosphamide. Blood samples were also collected on the 28th day to measure antibody titre.

Immunostimulation (%) was calculated as:

$$\frac{\text{Antibody titer of test} - \text{Antibody titer of vehicle control}}{\text{Antibody titer of test}} \times 100$$

The dose-response curve of percentage immunostimulation against log dose was plotted, and the half-maximal effect of the treatments was extrapolated from the regression line graph equation [17].

2.5. Antigen

The antigenic material used was sheep red blood cells. Fresh blood was collected from male sheep sacrificed at the local slaughterhouse. The sheep red blood cells (SRBCs) were washed three times with pyrogen-free normal saline (0.9% w/v NaCl) by centrifugation at 3000 x g for 10 minutes on each occasion. The washed SRBCs were adjusted to a concentration of 10^9 cells/mL. The RBC count of this suspension was determined by a hemocytometer using a Neubauer chamber.

2.6. Experimental design

2.6.1. Animal grouping

Animals (Mice) were divided into five groups of six mice each. Naïve control received 10 mg/kg Tween 20, and positive control (Reference drug) received 5.75 mg/kg of levomisole. Groups 3, 4 and 5 received ethyl acetate fractions doses in 93 (half of ED_{50}), 186 (ED_{50}) and 372 (double of ED_{50}) mg/kg as small, medium and high doses, respectively. Treatment lasted for 30 days.

2.7. *Determination of humoral antibody (HA) responses*

2.7.1. *Immunostimulation Assay*

The study was conducted with albino mice divided into control and treatment groups. The most active fraction (ethyl acetate fraction) of *D. bidens* was administered at different doses to their respective groups of six mice per group. Group 1 served as the vehicle control group and received 10 mL/kg of 5% Tween 20. Groups 2 - 4 were given the ED_{50} dose derived from the immunosuppressed experiment, half the ED_{50} dose, and double the ED_{50} dose (186, 93, 372 mg/kg) of the ethyl acetate fraction. Group 5 received the reference standard – levamisole at the dose of 5.75 mg/kg, which was also the ED_{50} dose of the reference standard derived from the immunosuppressed study. For humoral response, animals of all groups were challenged with 0.2 mL of SRBC i.p on the 23rd day and blood samples were collected on the 28th day, 6 h after the last treatment [18].

Immunostimulation (%) was calculated as:

$$\frac{\text{Antibody titer of test} - \text{Antibody titer of vehicle control}}{\text{Antibody titer of test}} \times 100$$

2.7.2. *Haemagglutinin Titre (HT) Assay*

Measurement of antibody titer by hemagglutination reaction was performed by using the method of Nworu et al. [19]. Blood samples were obtained from the retro-orbital plexus, and 25 µl of serum was serially diluted twofold in a 96-well microtiter plate using pyrogen-free sterile normal saline. The diluted sera were challenged with 25 µl of 1% (*v/v*) SRBCs in the plates and then incubated at 37°C for 1 h. The antibody titer was expressed in terms of maximum dilution, which gave a positive hemagglutination reaction. Antibody titres were expressed in a graded manner, the minimum dilution (1/2) being ranked as 1 (calculated as - $\log_2$ of the dilution factor).

2.7.3. *Humoral Antibody (HA) responses to ovalbumin*

The effect of the ethyl acetate fraction on humoral immune responses were determined in mice using

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ovalbumin (OVA) as the antigen in a homologous prime-boost strategy. Mice were randomized into five groups (n = 6) for use in these studies. Mice in Group I served as the negative control and were treated with only 10 mL/kg 5% Tween 20 and immunized on days 3 and 14 with 100 mg OVA/mouse injected into their hind footpads (50 mL/footpad). Group II mice were, in addition to this immunization with antigens on days 3 and 14, given 5.75 mg/kg Levamisole and served as the reference group. Mice in Groups III, IV, and V were given ethyl acetate fraction (186, 93, 372 mg/kg) daily and, similarly, immunized with OVA on days 3 and 14.

All mice were bled by a retro-orbital venous puncture on day 14 before receiving the boost immunization; the mice were also sampled on days 21 and 28 (7 and 14 d post-booster). Sera samples recovered each time were used to determine host primary and secondary humoral responses using ELISA [20].

The antibody titers elicited against OVA in the treated and control groups were estimated by enzyme-linked immunosorbent assay. ELISA plates - 96-Well Micro titer were coated with

100 mL of OVA (1mg/mL) in bicarbonate-coating buffer (pH 9.6) and incubated overnight at 4ºC. Unbound OVA was then washed off with ELISA wash buffer (PBS-T; containing 0.05% Tween-20 in 0.01M phosphate-buffered saline [PBS, pH 7.4]). Non-specific binding was blocked by the addition of 1% solution of bovine serum albumin in PBS to each well and the plate was incubated for 1 h at room temperature (RT). The wells were then washed again with PBS-T and then 100 mL of 1:20 dilutions of the sera samples were added to dedicated wells. The plates were incubated for 1 h at 37ºC before unbound serum proteins and other constituents were washed off using PBS-T.

To assess the antibody titer, 100 mL horseradish peroxidase (HRP)-conjugated goat anti-mouse total IgG, IgG1, or IgG2a secondary antibodies (BD Bioscience, Heidelberg, Germany) were added (each at 1:1000 dilution) to dedicated wells in the plates and the plates were then incubated another 1 h at RT. Thereafter, the unbound conjugates were washed off with PBS-T and 100 mL/well of freshly prepared ABTS substrate was added. The plate was incubated at RT for 15 min before the reaction in each well was stopped by the addition of 100 mL of peroxidase stop solution. The colour developed in each well was assessed at 405 nm using ELISA Plate Reader.

2.8. Determination of cellular immune function

To determine cellular immune function, the delayed-type hypersensitivity (DTH) response was evaluated in the extract-treated mice [21].

2.8.1. *Delayed-type hypersensitivity (DTH) response*

The effect of short-term supplementation of the ethyl acetate fraction on cell-mediated immune responses was assessed by determining the delayed-type hypersensitivity (DTH) response to ovalbumin in mice.

Mice were randomized into five groups (n = 6). Group 1 served as the vehicle control group and received 10 mL/kg of 5% Tween 20. Groups 2 - 4 were given the ED_{50} dose derived from the immunosuppressed experiment, half the ED_{50} dose, and double the ED_{50} dose (186, 93, 372 mg/kg) of the ethyl acetate fraction. Group 5 received the reference standard levamisole at the dose of 5.75 mg/kg, which was also the ED_{50} dose of the reference standard derived from the immunosuppressed study. Treatment was done per os daily. On day 3 of the study, all mice were primed by intraperitoneal injection with 100 mg ovalbumin (OVA; 1 mg/mL; Sigma-Aldrich, Germany) in normal saline. On day 13, the size of the left footpads of each mouse was determined using a high-precision digital micrometre screw gauge and then given a booster OVA immunization (100 mg in 50 mL volume) in the footpad. The thickness of the footpad of each mouse was then measured again 24 h later, and the DTH response was determined from the increase (swelling) relative to the initial footpad size and expressed as mean percent thickness/oedema.

2.9. Determination of non-specific immune function

For the determination of non-specific immune functions, the phagocytic activity of Macrophages (carbon clearance test) was studied.

2.9.1 *Phagocytic activity of reticuloendothelial system (carbon clearance test)*

The effect of the ethyl acetate fraction on the *in vivo* phagocytic activity of the reticuloendothelial system was determined using a carbon clearance test by Tripathi et al. [22]. Mice were randomized into five groups (n = 6) for the study. Group 1 was the vehicle

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control group and received 10 mL/kg of 5% Tween 20. Groups 2 - 4 were given the ED_{50} dose derived from the immunosuppressed experiment, half the ED_{50} dose, and double the ED_{50} dose (186, 93, 372 mg/kg) of the ethyl acetate fraction. Group 5 received the reference standard levamisole at the dose of 5.75 mg/kg, which was also the ED_{50} dose of the reference standard derived from the immunosuppressed study. Treatment was done per os daily. On day 8, 30 min after the last dose was administered to all mice, the hosts were given a single intravenous injection of carbon suspension (1:50 dilution of Indian ink) at 5 mL/kg body weight. Blood samples were drawn from the retro-orbital venous plexus before injection (0 min) and 15 min after injection of the carbon. Each blood sample (50 mL) was lysed with 4 mL of 0.1% sodium carbonate (Na_2CO_3) solution, and the optical density (OD) of the lysed sample was measured spectrophotometrically at 650nm.

The phagocytic index was then calculated as

$$k = \frac{([\ln OD15\ min] - [\ln OD0 min])([\ln OD15\ min] - [\ln OD0\ min])}{[t15\ min - t0\ min]}$$

Where, OD15 min and OD0 min are the optical densities at time t15 min and t0 min, respectively. Phagocytic indices were normalized to the mean phagocytic index for the untreated control group.

2.10. Statistical analysis

The study used SPSS 18.0 using independent tests like Student's t-test and ANOVA to compare groups, and the results are significant at p-value < 0.05.

3. Results

3.1. Phytochemical analysis and total phenolic content of D. bidens leaf extract and ethyl acetate fraction.

The phytochemical analysis of the extract and ethyl acetate fractions revealed the presence of flavonoids, saponins, Tannins, polysaccharides, alkaloids, and glycosides, while steroids and terpenoids are absent in the ethyl acetate fractions (Table 1). The total phenolic content of the ethyl acetate is recorded at 328.5 with a 40.32 % yield compared to the extract at 175.3 with a 2.56 % yield, as also shown in Table 1.

3.2. Effective dose (ED_{50}) estimation of D. bidens leaf extract and ethyl acetate fraction in immunosuppressive mice.

The effective doses of *D. bidens* leaf extract, ethyl acetate and water fractions were 301, 186 and 759 mg/kg, while the reference drug was 5.75mg/kg. Therefore, the extract and ethyl acetate showed dose-response activity at tested doses (50-1200 mg/kg), just like the reference drug (Levomisole, 5 – 120 mg/kg). Water fraction has the least effect, while ethyl acetate produced the highest therapeutic effect and the dose selected for further investigation (Fig. 1).

Table 1: Phytochemical analysis and total phenolic content of the extract and fractions

Phytocompounds	Extract	Ethyl acetate fraction
Flavonoids	+	+
Saponins	+	+
Tannins	+	+
Reducing sugar	+	+
Steroids	+	-
Terpenoids	+	-
Alkaloids	+	+
Glycosides	+	+
TPC (mgGAE/g)	175.3	328.2
Yield (%)	2.56[a]	40.32[b]

+ Present, - absent. a - calculated from 6kg pulverized leaves, b – calculated from 100 g extract. TPC = Total phenolic content

3.3. Effect of ethyl acetate fraction on immunocompetent Haemagglutinin titre (Humoral antigen) response in mice

Sheep red blood cell (SRBC) antigenic stimulation of immunocompetent mice evoked immunologic stimulation of anti-SRBC antibody production (Fig. 2a). Pre-treatment of the animals with the ethyl acetate fraction showed a dose-related response that reached a significant ($P<0.05$) level at the highest tested dose of 372 mg/kg compared to the vehicle control group. The immunostimulation recorded by the ethyl acetate fraction at this dose was higher (39.62%) compared to the 5.75 mg/kg dose of the reference drug, levamisole (32.56%) (Fig. 2b). Statistical comparison between the reference drug and ethyl acetate fraction treatment groups revealed no significant ($P>0.05$) difference.

3.4. Effect of humoral antibody responses to ovalbumin

The ethyl acetate fraction evoked ovalbumin-specific total IgG, IgG1 and IgG2a subtypes against ovalbumin antigenic challenge (Fig. 3). At first encounter (primary response), only 186 and 372 mg/kg of the ethyl acetate fraction showed significant stimulation of IgG antibody titre just like the reference drug – levamisole.

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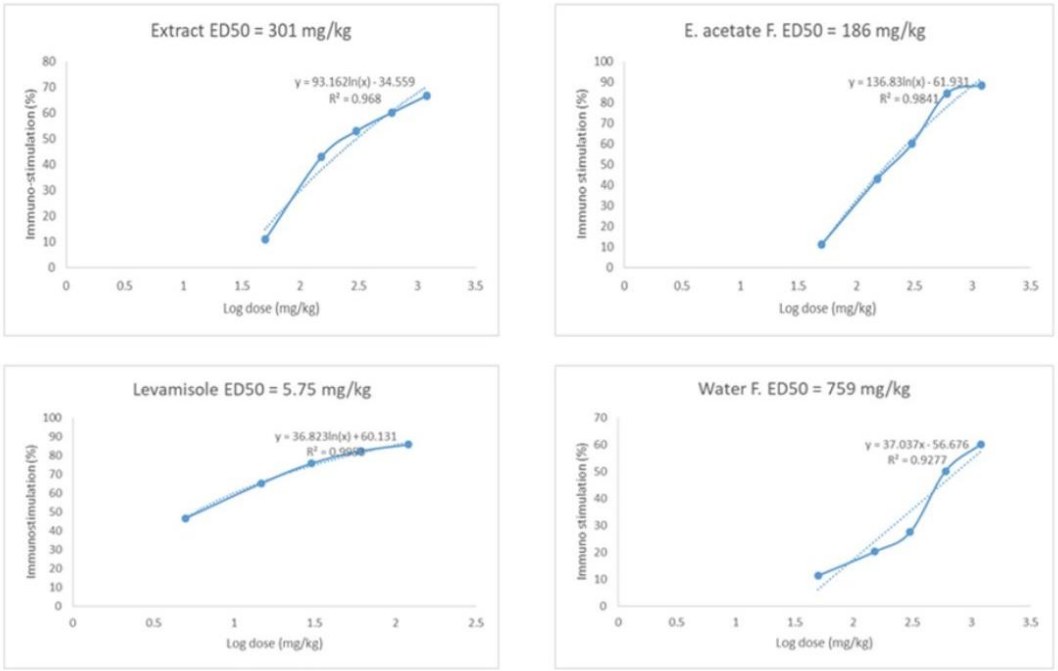

Figure 1. Dose-response curve for the determination of the effective dose of the extract and ethyl acetate fraction of *D. bidens leaves.*

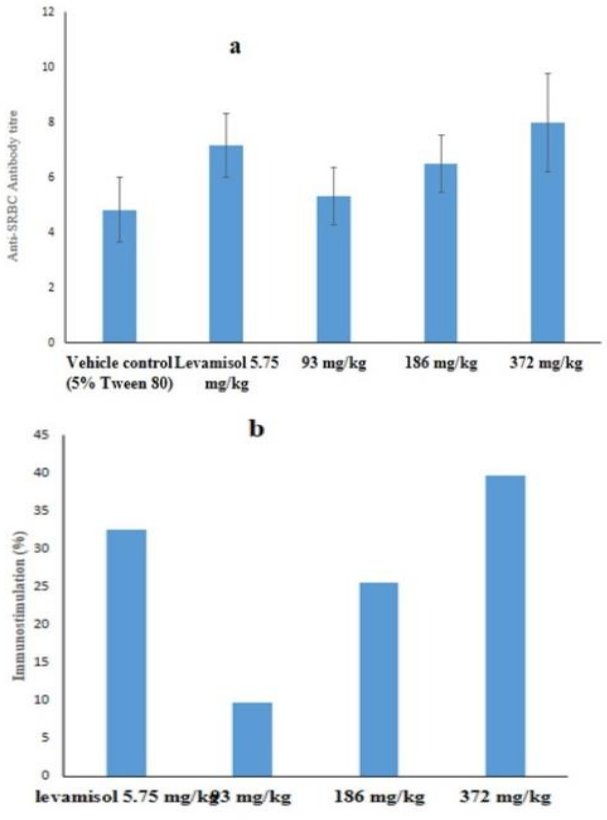

Figure 2. Immunostimulatory effect of the ethyl acetate fraction of *D. bidens* in immunocompetent animals.
(Where: * P<0.05 compared to the vehicle control group; # P<0.05 compared to the reference drug – levamisole 5.75 mg/kg; different letter alphabet (a, b, c) P < 0.05 compared to the doses of the ethyl acetate fraction).

The 7th-day and 14th-day post-boost response (secondary and tertiary) revealed that all the doses of ethyl acetate fraction recorded significant responses compared to a vehicle control group. All doses of the ethyl acetate fraction also recorded significant (P<0.05) primary, secondary and tertiary responses compared to a vehicle control group for IgG1 and IgG2a subtypes of IgG antibody. Compared to the response produced by primary exposure, higher responses were evoked after a booster dose of the antigen.

3.5. Effect of the ethyl acetate fraction on delayed-type hypersensitivity response (DTHR)

The ethyl acetate fraction of *D. bidens* evoked a delayed-type hypersensitivity response to ovalbumin (Fig. 4). A significant (P<0.05) dose-related effect was recorded compared to a vehicle control group. The responses evoked by the graded doses at 93, 186, and 372 mg/kg of the ethyl acetate fraction, differ significantly (P<0.05) from each other, as the lower dose, 93 mg/kg, evoked a low response, while the highest dose, 372 mg/kg produced high response compared to the vehicle control groups, and in turn significantly (P<0.05) differ from reference drug, levamisole.

3.6. Effect of ethyl acetate fraction on phagocytic activity (carbon clearance test).

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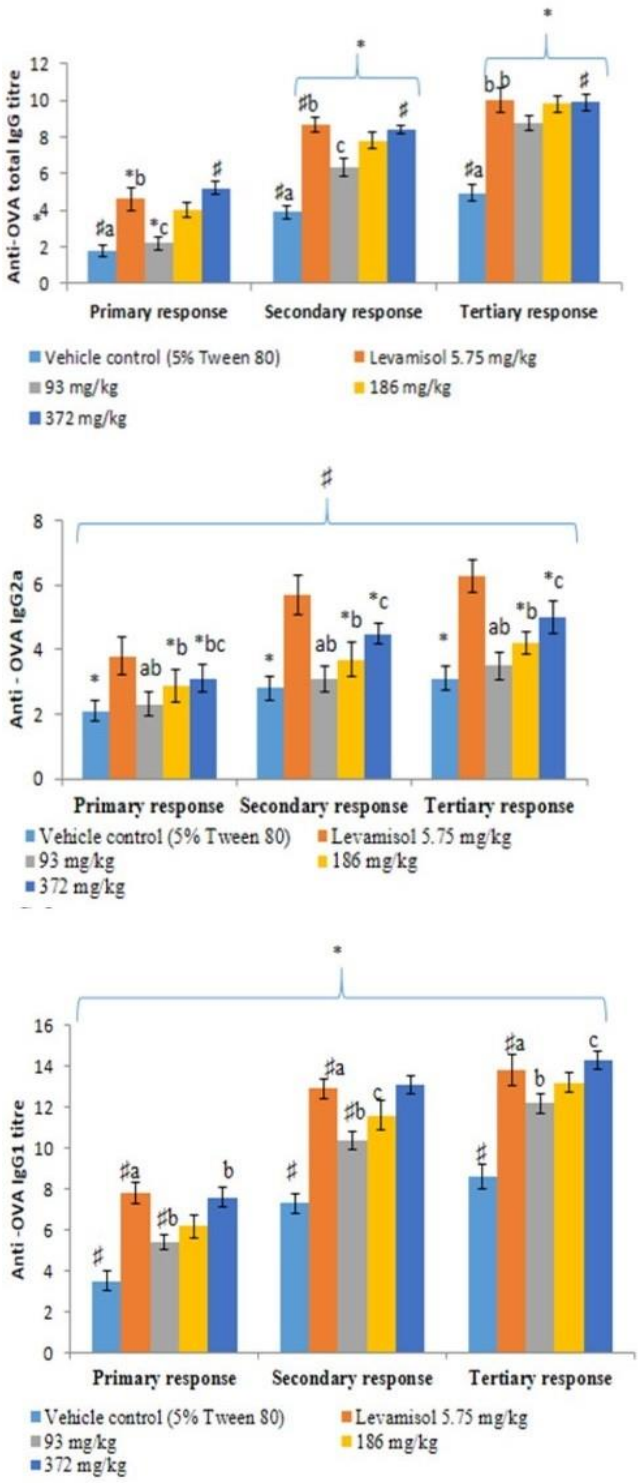

Figure 3. Effect of ethyl acetate fraction of *D. bidens* on Ovalbumin-specific antibody responses.
(Where: * P < 0.05 compared to the vehicle control group; # P < 0.05 compared to the reference drug – levamisole 5.75 mg/kg; different letter alphabet (a, b, c) P<0.05 compared to the doses of the ethyl acetate).

Administration of the ethyl acetate fraction of *D. bidens* produced a carbon clearance (phagocytic) effect (Fig. 5).

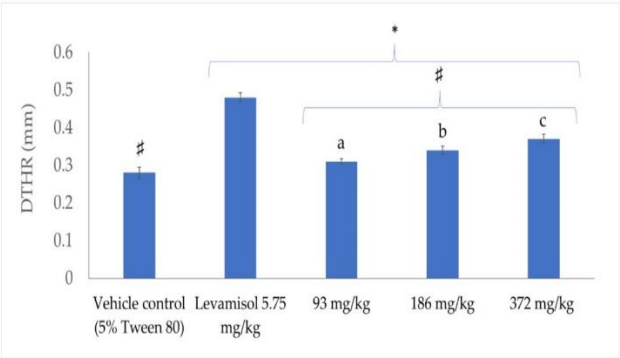

Figure 4. Delayed-type hypersensitivity response in mice treated with the ethyl acetate fraction of *D. bidens.*
(Where: * P<0.05 compared to the vehicle control group; # P<0.05 compared to the reference drug – levamisole 5.75 mg/kg; different letter alphabet (a, b, c) P < 0.05 compared to the doses of the ethyl acetate).

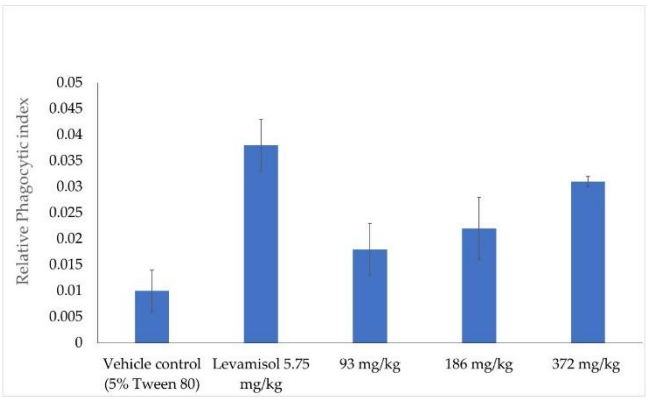

Figure 5. Effect of ethyl acetate fraction of *D. bidens* on phagocytic index of reticulo-endothelial system.
(Where: * P<0.05 compared to the vehicle control group; # P < 0.05 compared to the reference drug – levamisole 5.75 mg/kg; different letter alphabet (a, b, c) P < 0.05 compared to the doses of the ethyl acetate).

This effect was dose-related and significantly (P < 0.05) higher than that produced by the vehicle control group. Lower doses of the ethyl acetate fractions of 93 and 186 mg/kg produced significantly (P < 0.05) lower carbon clearance compared to the reference drug. However, at 372 mg/kg of the ethyl acetate fraction, similar effect was recorded with statistically non-significant (P>0.05) difference between both groups.

4. Discussion

Diaphananthe bidens (*D. bidens*) has been predominantly characterized as possessing anti-oxidant, anti-inflammatory, and anti-hyperglycemic properties in laboratory animals [2, 3] and has found

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application in traditional medicine for the treatment of rheumatoid arthritis and asthma [1, 3]. The anti-inflammatory effects observed in both *in vitro* and *in vivo* animal models are thoroughly documented and recognized [10]. In the present study, *Diaphananthe bidens* exhibited overall stimulatory effects on both specific and non-specific immune functions in mice. The stimulatory effects were observed following the pre-treatment of the animals with ethyl acetate fractions, which exhibited a dose-related response that attained significant (P<0.05) levels at the highest dose of 372 mg/kg, compared to the vehicle control. This was administered alongside sheep red blood cells, resulting in immunologic stimulation of anti-SRBC antibody production. This finding was in agreement with Raisuddin et al. [21] who showed that the extract of *Trigonella forenum* evoked immunological stimulation of sheep red blood cells antibody production in a dose-dependent manner, thus, an increase in dose might have increased the upward- regulation of immune functions. The humoral immune response to SRBC's antigen shows a significant (P<0.05) rise in HA antibody titres proportional to the ethyl acetate fraction of *D. bidens* leaf extract concentration. This suggests the ethyl acetate fraction of *D. bidens* leaf may stimulate lymphocytes, especially B lymphocytes, and other cells in the humoral immune response. Following the interaction between B lymphocytes and antigens, plasma cells generate antibodies, leading to their proliferation and differentiation into cells that secrete antibodies [23]. The designed antibodies attach to the antigen, enhancing its recognition by phagocytes, ultimately leading to its complete eradication [24].

Research conducted by Manayi et al. [25] and Arora et al. [26] demonstrated that *Echinaceae purpurea* and *Asparagus racemosus* respectively resulted in an elevation of both cellular and humoral immunity. This was evidenced by increased proliferation of T- and B-lymphocytes, as well as NK cells, alongside heightened activity of macrophages and neutrophils, thereby enhancing the body's defence mechanisms against bacterial and viral infections. In contrast to the above, Tilwari et al. [27] documented a suppressive influence on the humoral immune response in murine subjects following the administration of *Abrus precatorius*. The disparity can be ascribed to the inhibition of functional activity in antigen-presenting cells (APC), or the specific type and concentration of cytokines present within the microenvironment following lymphocyte activation.

The ethyl acetate fraction of *D. bidens* evoked a delayed-type hypersensitivity (DTH) response to ovalbumin, which caused significant (P<0.05) dose-related effects compared to vehicle control and the response significantly (P < 0.05) differed with ethyl acetate graded doses (93,186 and 372 mg/kg) from each other. This indicates that the plant extract exhibits immunostimulatory properties concerning the DTH response. The increase can be due to the extract's capacity to stimulate lymphocytes and various immune cells, consequently enhancing cell-mediated immunity [28]. In the context of DTH response, activated T cells play a crucial role in activating and recruiting additional macrophages via the secretion of lymphokines. This process enhances vascular permeability, induces vasodilatation, and contributes to the inflammatory response [29].

Earlier investigations, Dayanand et al. [30]; Gautam et al. [18], and Bin-Hafeez et al. [21] have similarly documented an enhancement in DTH response following the administration of botanical substances.

The primary phytochemicals identified in the ethyl fractions include polysaccharides, tannins, saponins, flavonoids, and alkaloids. Saponins, specifically, are characterized as agents that modulate the immune response [31]. It was proposed that certain constituents may exhibit mitogenic effects, subsequently resulting in stimulatory impacts on immunocompetent cells [32]. Certain phytochemicals exhibit antioxidant properties and may elicit immunostimulant effects, as numerous antioxidants have been documented to possess immunomodulatory characteristics [25-27].

The administration of the ethyl acetate fraction from *D. bidens* led to a significant (P < 0.05) carbon clearance effect, indicating phagocytic activity. This effect was dose-dependent and significantly higher than the vehicle control group. Reduced amounts of the ethyl acetate fraction resulted in a decrease in carbon clearance compared to the reference medication. A comparable effect was observed at a concentration of 372 mg/kg. The process of phagocytosis, along with the subsequent elimination of invading

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microorganisms by macrophages, represents the body's foremost line of defense against infections [36]. Macrophages play a crucial role in the immune system, functioning as phagocytic, microbicidal, and tumoricidal effector cells [29, 30]. Through their interaction with lymphocytes, macrophages play a crucial role in both the initiation and regulation of the immune response [39]. Plant extracts have been shown to possess immunomodulatory effects on nonspecific immunity [40]. Secondary metabolites, such as tannins present in the ethyl acetate fraction of *D. bidens*, have been shown to have nonspecific Immunomodulatory effects [33, 34]. The finding from the study is supported by previous research [35-38].

5. Conclusions

This present research showed that *Diaphananthe bidens* leaves possess immunomodulatory activity. The plant is already reported as anti-hyperglycemic, anti-inflammatory and anti-oxidant among others. Further research is necessary to isolate the phytochemicals responsible for its Immunomodulatory. However, based on literature and studies, saponins, polysaccharides and flavonoids are present in the plant extracts for candidates eliciting Immunomodulation.

Abbreviations

ED_{50}:	Median effective dose
LD_{50}:	Median lethal dose
ANOVA:	Analysis of variance
SRBC:	Sheep red blood cell
TPC:	Total phenolic content
Mg/GAE/g:	Milligram/Gallic acid Equivalent/gram
DTHR:	Delayed-type hypersensitivity response
I.P.:	Intraperitoneal injection

Ethics of approval and consent to participate

The animals used for the study of the immunomodulatory effect of the ethyl acetate fraction of *Diaphanathe bidens* leaf in mice were approved by the Nnamdi Azikiwe University Animal Research and Ethics Committee under the approval number NAU/AREC/2024/088.

Authors' contributions

Conceptualization, methodology, data collection and analysis. M.O.; Data interpretation and Visualization. U.H.O, D.L.A; Writing and submitting a manuscript. M.O; Supervision. I.S.M, E.C.O; Editing and approval for the final draft. E.C.O, I.S.M.

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Availability of data and materials

All data will be made available on request from the journal editor, following the policy.

Conflicts of interest

No conflict of interest to be disclosed.

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