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RESEARCH ARTICLE

Immunoactive profile of *Desmodium adscendens* L. Aqueous Extract in Mice

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ABSTRACT
In this paper we examine the pharmacological effects of Desmodium adscendens on some immunological parameters. This study, unlike any other, deals with the evaluation of impact on the acute, subacute, and subchronic oral administrations of different doses of the aqueous extract of D. adscendens, on both the cellular and humoral immunity. The flow cytometry technique was used to determine the number of granulocytes, monocytes, and total lymphocytes, particularly TCD4$^+$, TCD8$^+$ and NK cells. Furthermore, the ELISA method was used to measure the concentration of immunoglobulins (A, E and G). For the first time, the results clearly demonstrated that D. adscendens affects the immune system. Despite the fact that D. adscendens decreased the counts of the total lymphocytes, particularly TCD4$^+$, TCD8$^+$ and NK cells; it decreased the humoral immunity and increased the monocytes counts. Our findings ensure that D. adscendens has potentially strong effects on both the cellular and humoral immunity, thus explaining its extensive traditional uses.
KEYWORDS: *Desmodium adscendens L.; Immunoactive property; Leukocytes; Immunoglobulins; Flow cytometer; ELISA.*

INTRODUCTION

Alternative medicine is the general term for 'medicine and treatment that have not been verified scientifically or applied clinically in modern Western medicine' [1-3]. The range of alternative medicine varies widely to include traditional medicine and folk remedies as well as new therapies that are not covered by health insurance. It's a well known fact that the use of plants as medicines dates back to the earliest years of man's evolution [4,5], though financial problems pose a hindrance bloc for a huge segment of people, throughout the world, to access such remedies. According to the National Institutes of Health, over 60% of Americans use complementary and alternative medicine with ~20% using natural products or specific botanical formulations to supplement their health care needs [6]. A Large number of these plants, and their isolated constituents, have shown beneficial therapeutic effects, including anti-oxidative, anti-inflammatory, anti-cancer, anti-microbial, and immunomodulatory effects [5-12]. Furthermore, Ethnopharmacological studies have shown that people in different cultures and places have used the same plants for similar medical problems.

Desmodium adscendens (Sw.) DC. (Fabaceae) is a widespread plant, but is mostly found in Latin America [13], Africa [14] and in India equatorial areas [15]. It is used as medicine in many parts of the world and has been regarded to possess varied medicinal properties.100 g of this plant contains 10.98 g of carbohydrate, 0.81 g of protein and 0.19 g of lipids and. The calorie intake of this 100 g is 49 Kcal [16]. In Brazilian traditional medicine, the dried leaves of *D. adscendens* are used to treat leucorrhea, body aches and pains, ovarian inflammations, excessive urination, gonorrhea and diarrhoeas [17].

In the African traditional medicine, *D. adscendens* is also widely used. For example, in Ghana, the plant decoction is prescribed by healers to treat asthma and other diseases associated with smooth muscle contraction [14]. This characteristic of the *D. adscendens* has been proved to inhibit contraction of the guinea pig's ileum and airways [18-20]. In addition, its traditional use in the hepatic affection, particularly viral hepatitis, was recorded and verified [21]. Some anti-allergic effects were

also verified *in vivo* [20,22]. Besides these uses, healers in Congo have exploited *D. adscendens* as a cure for several diseases, including fever, pain and epilepsy [23]. It has been suggested that the action mechanism of the plant is due to the depletion of the histamine stocks [19]; the inhibition of the ciclooxygenase and lipooxygenase enzymes [20]; the increase of the prostaglandin synthesis, PGE_2 and PGF_{2a}; the opening of the BKCa channels [24], and the inhibition of cytochrome P450 NADPH-dependent arachidonic acid metabolism [25].

We herein investigate the effects of the different doses of aqueous extract of *D. adscendens* on the cellular and humoral immunity of mice. The number of the total lymphocytes, T lymphocytes ($CD4^+$, $CD8^+$, NK), granulocytes and monocytes was determined by using flow cytometry technique; and the immunoglobulin concentrations (IgA, IgE and IgG) were measured by ELISA technique. According to our knowledge, there is no study that has demonstrated the immunoactive property of the *D adscendens* L. on these immunological parameters.

MATERIALS AND METHODS

Plant Material and Reagents

Dried leaves and stems of *D. adscendens* were obtained from Nigeria. Halothane was obtained from Belmont Laboratories, France. Heparin was obtained from Sanafi-Synthelabo, France. Anti mouse antiCD4, CD8 and NK, lysing solution and CellWASH solution were obtained from Becton Dickinson (BD, USA). The monoclonal IgA, IgE and anti IgE were obtained from Interchim, France. The monoclonal IgG, anti IgG, anti IgA, Phosphate Buffered Saline (PBS) 1X and Carbonate-bicarbonate buffer were obtained from Sigma Co., St. Louis, MO, USA. Tween 20 and Sulfuric Acid were purchased from Roth. Bovine Serum Albumin (BSA) and 3, 3, 5, 5' Tetramethylbenzidine (TMB) were obtained from Merck, Calbiochem, Germany. The 96-well plates were obtained from Nunclon, Delta, Denmark.

Experimental Animals

We used Swiss albino male mice (OF1), 9 weeks old at the time of reception from the breeder (Charles River, France) ranging in weight from 35–40 g. The animals were housed with a 12-h light: 12-h dark schedule (lights on at 8:00 p.m.) with free access to water and food (SDS Dietex - France) and maintained at a constant temperature (21±2 °C) and a relative humidity of 55±10%. Experiments began after a 2-week period of acclimatization. All the procedures applied in the experiment, on these mice, were in accordance with the European Communities Council Directive of November 24th, 1986 (86/609/EEC).

Preparation of Aqueous Extract

The extract used in the experiment was prepared from the dried *D. adscendens* plant, according to the traditional method followed in the Nigerian medicine. The dried leaves and stems (200 g) were crudely crushed. A decoction was performed for an hour in boiled distilled water (1 l). It was then filtered and dried under reduced pressure, and later transformed into a dry product using a lyophilisator (ALPHA 1-2 LD, Fisher Bioblock). The obtained yield was 24.2 g. This product was stored in opaque bottles.

Oral Drug Administration

The extract was administered *per os* using feeder needles (Letica – Spain). All doses were injected in a volume of 10 mL/kg, and were prepared only in distilled water, not any other solvent. Control group was administered the distilled water *per os*.

Study Design

The effects of five doses (10, 30, 60, 240 and 500 mg/kg body weight) of the aqueous extract of *D. adscendens,* administered orally, on leukocytes subsets, and the immunoglobulin concentrations were tested after several treatment durations: acute (one day, D1), subacute (seven days, D7) and subchronic treatment (twenty-one days, D21). However, distilled water was administered orally to the control group. Each dose was administered to ten mice. Administrations were done on a daily basis, one hour after the light was put out. The cycle of activity in mice or rats is nocturnal. Since rodents cannot perceive the white light frequencies, most of the behavioral and ethological studies were performed under the red light that allows the experimenters to carry out the protocol without interfering with the rodent's activity cycle. Measurements of the various immunological parameters were done one hour after the drug's administration.

Toxicological Evaluation

To evaluate acute, subacute and subchronic toxicity of *D. adscendens*, several doses (20, 100, 200, 500, 2500, 5000 and 10000 mg/kg) of the plant decoction were administered orally to different groups of mice.

Acute toxicity tests were performed. Observations were made 30 min, 1, 2, 3, 4, and 24 hour(s) after introducing the treatment. Subacute toxicity was performed four weeks after a single treatment in concurrence with daily observations and weekly weighing of the mice. Subchronic toxicity was performed four weeks, with daily administrations and observations, weekly weighing, and anatomical-pathological examinations at the end of the treatment period.

Immunological Evaluation

Leukocyte Subsets Analyzed by Flow Cytometry

Flow cytometry is a technique used for the individual characterization of particles suspended in a liquid environment after their luminous excitation. Three-parameter flow cytometric analysis, using direct immunofluorescent staining of whole blood, was performed on a FACScan flow cytometer (Becton Dickinson) utilizing fluorescein isothiocyanate (FITC), phycoerythrin (PE), and peridinin chlorophyl protein (PerCP).In order to label surface molecules which identify CD4, CD8 and NK cells, blood was incubated with 5 µl of three monoclonal antibodies (i) anti-mouse anti CD4-PerCP, (ii) anti-mouse anti CD8-PE and (iii) anti-mouse anti NK-FITC respectively. The number of cells was calculated using CellQuest software (BD Bioscience). A total of 10 000 cells were analyzed for all samples.

Measurement of immunoglobulins by ELISA

The total immunoglobulin concentrations, which were given in µg/mL, were determined and quantified as tested in the mouse's serum by ELISA method. The wells of 96-well plates were read at 460 nm using a Bio Tek 312e micro plate reader (Bio Tek, Winooski, VT, USA). Ranges of the ELISA standard curve were 3.9-125 µg/mL; 12.5-200 µg/mL and 3.125-200 µg/mL for IgG, IgA and IgE respectively.

Statistical Analysis

Data following Gaussian distribution were analyzed by Student t-test and reported as mean ± S.E.M. For all statistical evaluations, the level of significance, between the control and the treated groups, was set at $P<0.05$. All statistical analyses were carried out using the Statview® 4.5 statistical package (Abacus Concepts, Inc).

RESULTS

Preliminary chemical screening

The qualitative chemical analysis of the aqueous extract of *D. adscendens* showed that preliminary alkaloid tests were positive for both tertiary and quaternary alkaloids according to Mayer's and Dragendorff's reagents. The screening for saponin component showed positive results with $FeCl_2$ and $HgCl_2$.Furthermore, the phytochemical screening of this plant showed the presence of flavanoids.

Plant toxicology

Acute, subacute and subchronic treatments, with different doses of *D. adscendens* on mice have shown, during the observation period, no signs of significant toxicities or death (data not shown). All mice survived the study. Thus, it appears that the LD50 of these doses, in mice, is more than 10 g/kg.

Effects of the acute treatment (D1) on granulocytes and monocytes counts

Results did not show significant differences as to the numbers of granulocytes and monocytes after an acute treatment, of mice, with different doses of the plant when compared with the results obtained from the control group (Table 1).

Effects of the acute treatment (D1) on total lymphocytes, $TCD4^+$, $TCD8^+$ and NK cells

All doses of the *D. adscendens* (10, 30, 60, 240 and 500 mg/kg) have provoked a significant decrease of the total lymphocytes, $TCD4^+$, $TCD8^+$ and NK counts in comparison to the control group (Table 1).

Effects of the acute treatment (D1) on the concentration of the Immunoglobulins (A, E and G)

After a D1 treatment, our results revealed that all doses of *D. adscendens* have provoked a significantly decrease in the concentrations of the IgA, IgE and IgG as compared to those of the control group (Table 4).

Effects of the subacute treatment (D7) on Total Lymphocytes, $TCD4^+$, $TCD8^+$ counts

Seven days of treatment by different doses of *D. adscendens* showed that no significant differences in the total lymphocytes, $TCD4^+$ and $TCD8^+$ ($P>0.05$) as compared with those obtained from the control group (Table 2).

Effects of the subacute treatment (D7) on the granulocytes and NK cells counts

Results revealed that after a D7 treatment with *D. adscendens* of 500 mg/kg dosage, a significant decrease was apparent in the number of the granulocytes as compared with those of the control group (Table 2). The level of significance was ($P<0.02$). Doses of 240 and 500 mg/kg have significantly decreased the number of NK cells versus the results obtained from the control group ($P<0.0048$) (Table 2).

Effects of the subacute treatment (D7) on the monocytes counts

Except for the 240 and 500 mg/kg doses, the *D. adscendens* has provoked a significant increase in the number of monocytes as compared with the control group ($P<0.05$) (Table 2).

Effects of the subacute treatment (D7) on the concentration of the Immunoglobulins (A and E)

Concerning the antibody production after a D7 treatment, all doses of *D. adscendens* did not affect the concentration of IgG as compared with those of the control group ($P>0.05$) (Table 4). Results obtained revealed that all doses of *D. adscendens* induced a significant decrease in the concentrations of IgA and IgE ($P<0.05$) versus results obtained from the control group (Table 4).

Effects of the Subchronic Treatment (D21) on granulocytes Counts

Results obtained after a D21 treatment, with all doses of *D. adscendens,* did not show significant differences pertaining to the granulocytes, as compared with the control group (Table 3).

Effects of the Subchronic Treatment (D21) on the Total Lymphocytes, $TCD4^+$, $TCD8^+$, NK and Monocytes Counts

The *D. adscendens* at the doses of 10, 60 and 500 mg/kg, has shown significant differences in the total lymphocytes numbers ($P<0.05$) when compared with the control group (Table 3). All doses of *D. adscendens* have shown significant differences in the NK cells numbers ($P<0.05$) when compared with the control group (Table 3). The 10 and 30 mg/kg doses have shown significant differences in the $TCD8^+$ numbers ($P<0.05$) when compared with the control group (Table 3). Concerning the $TCD4^+$, only the 10 and 500 mg/kg doses have shown significant differences ($P<0.05$) when compared with the control group (Table 3).

D. adscendens have provoked a significant increase in the monocytes at the 60, 250 and 500 mg/kg doses as compared with the results obtained from the control group (Table 3).

Effects of the Subchronic Treatment (D21) on the Concentration of the Immunoglobulins (A, E and G)

As shown in Table 4, our obtained results revealed that significant differences in the antibody production between groups (control and treated group) were observed ($P<0.05$).

D. adscendens at 60 mg/kg and 500 mg/kg dosage provoked a significant increase in the concentrations of the IgA and IgG respectively versus findings of the control group ($P<0.05$) (Table 4).

The IgE concentrations significantly decreased ($P<0.05$) after a D21 treatment with different doses of *D. adscendens* when compared with the control group (Table 4).

Table 1. Effect of *D. adscendens* on the count of granulocytes, monocytes, total lymphocytes, TCD4, TCD8 and NK cells after one day of treatment

	D1					
	Lymphocytes	**Granulocytes**	**Monocytes**	**TCD4**	**TCD8**	**NK**
Control	4081±360	2134±307	67±11	1965±180	518±45	453±47
10	2159±307 **	1697±378	50±20	1238±161 *	288±45 **	149±24 ***
30	1843±212 ***	2172±470	56±10	962±111 ***	219±23 ***	193±31 ***
60	1918±182 ***	1599±535	33±8 *	1057±101 **	238±27 ***	140±41 ***
240	2192±317 **	1490±395	37±12	1191±224 *	298±53 **	118±17 ***
500	2567±331 *	1511±243	106±25	1349±216 *	324±42 *	230±67 *

The aqueous extract of *D. adscendens* was administered *per os* at different doses, n = 10. Each datum represents the mean ± S.E.M.; *$P<0.05$; **$P<0.01$; ***$P<0.001$. Statistical analyses were performed by Student-t test.

DISCUSSION

Myriad compounds from nature have recently been investigated as to their capabilities for immune stimulation or suppression, for usage in infection, autoimmunity, allergy and organ transplant facilitation [26,27]. For the first time, this study investigated the influence of *D. adscendens* on the immune system, especially on granulocytes, monocytes, total lymphocytes and their subsets. For quantitative evaluation, flow cytometry and ELISA assessments were used to determine the leukocytic population's counts and the immunoglobulins concentrations.

Table 2. Effect of *D. adscendens* on the count of granulocytes, monocytes, total lymphocytes, TCD4, TCD8 and NK cells after seven days of treatment

	D7					
	Lymphocytes	**Granulocytes**	**Monocytes**	**TCD4**	**TCD8**	**NK**
Control	4081±360	2134±307	67±11	1965±180	518±45	453±47
10	3863±474	1538±337	134±21 *	1729±239	523±69	323±53
30	3934±332	2307±513	121±10 *	1991±221	577±75	467±43
60	4160±290	1602±363	157±25 **	1879±175	550±29	477±54
240	3524±138	2020±153	97±10	1854±116	501±34	198±16 **
500	4517±298	900±135 *	115±11	2177±184	534±37	210±20 **

The aqueous extract of *D. adscendens* was administered *per os* at different doses, n = 10. Each datum represents the mean ± S.E.M.; **P<0.05; **P<0.01; ***P<0.001*. Statistical analyses were performed by Student-t.

Table 3. Effect of *D. adscendens* on the count of granulocytes, monocytes, total lymphocytes, TCD4, TCD8 and NK cells after twenty-one days of treatment.

	D21					
	Lymphocytes	**Granulocytes**	**Monocytes**	**TCD4**	**TCD8**	**NK**
Control	4081±360	2134±307	67±11	1965±180	518±45	453±47
10	3322±401 **	1593±300	140±22 *	1593±211 *	373±47 *	148±28 *
30	4244±374	1336±184	144±16 *	1974±124	396±43 *	220±55 *
60	3783±307 *	2069±384	232±36 ***	1912±134	369±33	224±51*
240	4925±290	1795±343	159±18 *	2280±132	555±50	196±35 *
500	3434±419 **	2178±202	158±17 *	1768±183 *	423±43	222±64 *

The aqueous extract of *D. adscendens* was administered *per os* at different doses, n = 10. Each datum represents the mean ± S.E.M.; **P<0.05; **P<0.01; ***P<0.001*. Statistical analyses were performed by Student-t test.

Table 4. Effect of *D. adscendens* on the immunoglobulin concentrations after three weeks of treatment

	D1			D7			D21		
	IgA	**IgE**	**IgG**	**IgA**	**IgE**	**IgG**	**IgA**	**IgE**	**IgG**
Control	19.5±3	15±3	2.5±0.3	27±6	14.9±2.6	3.6±0.5	22±6	10±2	3±0.4
10	7.5±0.7 *	5±0.7 *	1.2±0.008 *	15±1**	3.9±0.3***	2.5±0.2	17±2	4±0.4***	5±1.5
30	6.7±0.2 *	6±0.5	1.2±0.008 *	16±3 *	4±0.5***	5.2±1.3	22±3	4±0.3***	6±2.7
60	6.5±0.4 *	6±0.36	1.3±0.003 *	15±1**	4.08±0.34***	4.67±0.8	33±4 *	6±0.8*	5±0.9
240	7.04±0.5 *	6±0.4 *	1.2±0.04 *	20±3	3.84±0.18***	4.12±0.4	12±2	3±0.2***	2±0.2
500	6.4±0.3 *	7±0.67	1.3±0.02 *	18±2 *	3.82±0.25***	3.7±0.5	27±5	4±0.3***	10±2**

The aqueous extract of *D. adscendens* was administered *per os* at different doses, n = 10. Each datum represents the mean ± S.E.M.; **P<0.05; **P<0.01; ***P<0.001*. Statistical analyses were performed by Student-t test.

Aqueous extract of *D. adscendens* contributes in general to an immune system depressant activity as indicated by the decreased concentrations of immunoglobulins and leukocytes subsets counts, except for monocytes. Our results demonstrated that acute and subchronic oral administrations of the aqueous extract of *D. adscendens* have induced a significant decrease in the counts of total lymphocytes, $TCD4^+$, $TCD8^+$ and NK cells. However, the NK cells counts remained relatively low throughout the experiment. Moreover, the acute treatment (D1) with *D. adscendens*, at different doses, did not influence the granulocytes number. By contrast, subacute treatment (D7) by the 500 mg/kg dose had significantly decreased their number. These granulocytes nearly restored their normal

value level at D21. Concerning the monocytes, *D. adscendens* did not have an immediate impact on them. However, after subacute and subchronic treatments, their counts increased significantly. This increase was two to three times higher than the normal values. It seems that *D. adscendens* act as a pacemaker to this population. In order that the immune system mounts an adequate defense, it must produce more T cells, through a process of differentiation and proliferation. Monocytes in peripheral tissues, upon antigen encounter, secrete monocyte growth factor, which activates monocytes to develop into macrophages [28]. Taking into account the role of the monocytes in the immune defense of the organism, and their ability to transform into macrophages, this plant's extract, in different doses, shows a stimulant effect that transforms these monocytes to macrophages. It seems that most of the plants showing immunomodulatory properties have major effects on nonspecific immunity, i.e. on macrophages functions [29]. It is a well known fact that the activated macrophages didn't only show increased phagocytosis but it also showed the inception of tumor necrosis factor-α, interferon-α and β, which may either have direct effecter functions or could mediate the effecter response of natural killer cells, and generate the cytotoxic T lymphocytes [30-32].

On the other hand, our results clearly showed that the total lymphocytes, including $TCD4^+$, $TCD8^+$ and NK counts, were significantly decreased after acute and subchronic treatments with different doses of *D. adscendens*. In view of these findings, it is possible to suggest that *D. adscendens* inhibits a part of the cellular immunity. In this context, it seems that our findings could explain some of the traditional uses of *D. adscendens* against several diseases, such as fever which increases the lymphocytes' efficiency [23].

The results from our study concerning the effects of *D. adscendens* on humoral immunity, showed that treatments (D1, D7 and D21), by the different doses, have induced a reduction in the concentrations of IgA, IgE and IgG. However, the doses of 60 mg/kg and 500 mg/kg have significantly increased the concentrations of IgA and IgG respectively, but only after three weeks of treatment (D21).

A balance between reciprocal responses, TH_1 and TH_2, is necessary for the immune system to achieve homeostasis. Each division, humoral immunity and cell-mediated immunity, has the ability to suppress the other's functioning, through inhibitory pathways. Also, there is evidence that each response is capable of self-inhibition, through negative feedback, when their respective cytokine production is high. This constitutes a system of checks-and-balances which ensures that neither type of immune response is uncontrollable [28]. Accordingly, we hypothesized that *D. adscendens* is a plant that has a strong effect on the immune system (humoral and cellular immunity), but these effects are not completely elucidated. Our present results are consistent with the findings of previous studies in which the anti allergic and anti asthmatic properties of *D. adscendens* have been demonstrated [14,20,22], since acute, subacute and subchronic treatments with this plant have significantly decreased the IgE level. Therefore, *D. adscendens* may inhibit the onset of the hyper-sensibility reactions. In fact, generally elevated levels of IgE indicate an IgE-mediated hypersensitivity responsible for allergic reactions by sensitizing and activating of the mastocytes, and which, in turn, release the inflammatory mediators (histamine and serotonin…). Patients with atopic allergic diseases, such as atopic asthma, atopic dermatitis, and hay fever, have shown an increase in the total IgE levels in the blood. Needless to say, *D. adscendens* may be effective against some parasitic diseases, such as hookworm and certain clinical disorders, including aspergillosis, which are responsible for the increased level of IgE [33-37].

In conclusion, for the first time, we have demonstrated that *D. adscendens* has an immunoactive property. The acute and subchronic oral administrations of *D. adscendens* have shown an immunoactive profile *via* their impact on both cellular and humoral immunity in mice. The depressive effect on the cellular immunity was, in general, statistically significant with respect to the total lymphocytes, including $TCD4^+$, $TCD8^+$ and NK cells. In addition, *D. adscendens* exhibited a stimulant effect on monocytes. On the other hand, the depressive effect was also observed in the humoral immunity, as pertaining to the IgE, IgA and IgG concentrations. However, our results cannot exclude the effect of *D. adscendens* on the redistribution of leucocytes from the blood to organs such as lymph nodes and skin. As the high level of IgE is often indicative of allergic reactions, *D. adscendens* may have an anti allergic-like effect, and a protective effect against autoimmune diseases and viral infections. Therefore, in a future study, the identification and the characterization of the active molecules in *D. adscendens* will be made.

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