International Journal for Research in Applied Sciences and Biotechnology

ISSN: 2349-8889
Volume-8, Issue-5 (September 2021)

www.ijrasb.com

https://doi.org/10.31033/ijrasb.8.5.10

Evaluation of Immunomodulatory Activity of Extract and Fractions of *Sida acuta* Burm. f. Leaves in Mice and GC-MS Analysis of n-Hexane Fraction

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ABSTRACT

The leaves of *Sida acuta* Burm. f. (Malvaceae) has been reported to possess potent anti-inflammatory, anti-plasmodial and anti-microbial activities. The relationship of these bioactivities and immune responses leads to the evaluation of the immunomodulatory activity of *Sida acuta* Burm. f. leaves extract and fractions. This study was done to determine the immunomodulatory activity and chemical study of methanol leave extract and fractions of *Sida acuta* Burm. f. The immunomodulatory evaluation was done by *invivo* Delay-Type Hypersensitivity reaction (DTHR) in the body and *in vitro* measurement of phagocytosis of killed *Candida albicans* by the phagocyte polymorphonuclear leucocytes using slide method. Acute toxicity, phytochemical and GC-MS analysis were also performed. The DTHR tested in the blood with T-cells in mice showed that the extract and its fractions caused a delayed hypersensitivity response in 24hrs which was very significant ($P < 0.05$) in the n-hexane fraction of the extract when compared to the control group at the dose of 100mg/kg. The *in vitro* studies showed a very significant difference ($P < 0.05$) in the positive control group (LEVA) at a concentration of 50, 100 and 200µg/ml, in crude extract (SrE) at concentrations of 50, 100 and 200µg/ml, n-hexane fraction 50, 100 and 200µg/ml, Ethyl acetate fraction at 200µg/ml and Absolute methanol fraction at 100µg/ml and also have high percentage phagocytic stimulation (PPS). The acute toxicity test did not cause clinical signs or death within 24hours post-treatment in all doses tested and the highest dose of 5000mg/kg. Phytochemical analysis revealed the presence of flavonoids, saponins, alkaloids, triterpenoids, tannins, steroids, and cardiac glycosides. GC-MS analysis of fraction with the highest activity was carried out on n-hexane fraction which showed the presence of some compounds like hexadecanoic acid, 2-hydroxy-1 (hydroxymethyl) ethyl ester, 3,4-seco-5alpha-cholestan-3-oic acid,4-hydroxy-4-methyl epsilon-lactone, (4R), 2- pentadecanone,6, 10, 14-trimethyl, 9,12-octadecadienoic acid, methyl ester, methyl stearate, cyclopentane tridecanoic acid, methyl ester, cyclopropane butanoic acid, 2-[[2-[[2-[(2-pentylcyclopropyl) methyl]cyclopropyl] methyl] methyl ester, etc. which have known bioactivity as immunomodulation showing that the crude extract and fractions of *Sida acuta* Burm. f. leaves have an immunostimulatory action and can serve as a drug candidate for immunomodulatory activity.

Keywords- Delayed-Type Hypersensitivity, *Sida acuta* Burm. f., Immunomodulation, Phagocytosis

I. INTRODUCTION

The reason for many diseases like cancer, allergy, asthma, arthritis, and infections is as a result of immune system impairment. When there is a decrease immune function in a patient, it is dependent on the low phagocytic activity of the neutrophil, macrophages, and other immune cells to their ability to fight bacteria when they are affected, which leads to several cases of infection on the host. [10].

The Immune system is made up of interconnected cells, proteins, active organs, and tissues found in all parts of the body, which our scientists believe that they help in fighting / curing diseases and infections. Our bodies are always prone to infection by various infections organisms like bacteria, viruses, parasites, foreign cells, etc. it is activated to protect the entire body from various disease-causing organisms that are around us. Hence, immune system components move around the body to search for invading organisms and elicit their response where needed. They ward off harmful or allergenic substances that enter through mucosal surfaces, also have the ability to differentiate one's tissue from non-self-tissues by identifying proteins that are attached on the peripherals of cells, these markers help the immune system to ignore its tissues and fight or destroy the foreign ones [7].

The immune system is also defined as a combination of a variety of biochemical fluids and bio reactions that work to prevent the whole system/ host body from invading microbe [18]. The disorders of the immune system involve allergic diseases, immunodeficiency diseases, cancers of the immune system, autoimmune diseases, graft- versus host diseases. Immunomodulatory drugs can be used to solve this problem by boosting the immune system [9]. The natural immune modulators act to fortify the weak immune system and moderate that is overactive [27]. Some medicinal herbs that possess immunomodulatory

activity include: *Actinidia macrosperma, Aesculus indica, Allium sativum, Aloe vera, Andrographis paniculata, Baliospermum montanum*, etc. and are used as drugs that serve as agents that could intensify the body's resistance against infections. These drugs are most commonly used for autoimmune disease, allergic conditions, cancer, Acquired immune Deficiency Syndrome, Viral infections, etc. [14]. The phytochemical constituents like alkaloids, terpenoids, steroids, proteins, and polysaccharides are considered to show this immunomodulatory property [10].

Pharmacological studies have confirmed that *Sida acuta* has several biological effects including antimalarial, antimicrobial, hepatoprotective, antioxidant, antidiabetic, anti-inflammatory, abortifacient, anticancerous, analgesic, nephroprotective, cardioprotective, neuroprotective, antiviral, and antiulcer [12]. *Sida acuta* has not yet been reported scientifically for any of its immunomodulatory activity. However, plants that belong to the Malvacea family have been studied in wide scope for immunomodulatory activity. The study was done to evaluate the immunomodulatory effect of methanol leaf extract of *Sida acuta* using measurement of phagocytosis and Delay type hypersensitivity reaction.

Fig 1.1: Diagram *of Sida acuta* Burm. f. (Leaves).

II. MATERIALS & METHODS

Plants collection and authentication

Sida acuta fresh leaves were gathered from farmlands at Oduke-Okwe village Asaba, in Oshimili south L. G. A of Delta State, in April 2019. They were identified and authenticated by Mr. Felix Nwafor, a plant taxonomist of Pharmacognosy and Environmental Medicines Department, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria. A voucher specimen has been placed in the herbarium under the number PCG/UNN/0320.

Extraction

The leaves were picked off the stem, shade dried at normal room temperature, and pulverized to a coarse powder using an electrical grinder. The pulverized plant samples were weighed using an electric analytical weighing balance and then macerated with aqueous methanol. The mixture was shaken vigorously and from time to time after which it was allowed to stand for 72hrs, while straining out the yield and replacing with new solvent every 24hrs till the yield turns colourless, after 72hrs. It was finally strained using a muslin cloth and then filtered with Whatmann filter paper number 1. The dark green filtrate was concentrated with a rotary evaporator, and further air-dried to yield a viscous dark greenish methanol extract of 47.18g and the percentage yield of the extract was calculated to be approximately 8% yield and the extract was stored in the refrigerator and used for pharmacological screening.

The quantity of plant sample collected was 590g and the weight of plant extract was 47.18g

$$\%\text{Yield}\left(\frac{w}{w}\right) = \frac{\text{Weight of plant extract}}{\text{Weight of plant material}} \times 100$$

Fractionation of Crude Extract

Fractionation of crude extract was done using different solvents of n-hexane, ethyl acetate, absolute methanol, and distilled water according to their polarity to get each fraction of the solvent.

Qualitative phytochemical analysis of plant crude extract and fractions of Sida acuta Burm. f.

The preliminary phytochemical tests were done at the Department of Pharmaceutical and Medicinal Chemistry, University of Nigeria, Nsukka by the standard method according to the procedures outlined by the literature. Phytochemical analysis was carried out to check and identify the active constituents of the crude methanolic leave extract, Absolute methanol, n-hexane, aqueous, and ethyl acetate fractions of *Sida acuta* Burm. f.

Drug preparation

Accurate weight of methanolic crude extract and fractions of *Sida acuta* Burm. f. leaves were dissolved in 1:4 of tween 80 and distilled water. Stock solutions were prepared; the proper volume of the drug was administered orally by selecting accurate doses and concentrations of the stock solution. Normal saline served as the negative control whereas the Levamisole served as the positive control. All chemicals, drugs, and reagents used in this research were of analytical grade, supplied by Sigma-Aldrich Inc, St. Louis, U.S.A. and Nigerian-German Chemicals, PLC.

Animals used for the study

Swiss albino mice of either mouse weighing 18-22g were procured from animal house, University of Nigeria, Nsukka. were divided into twelve (XII) groups of five animals each, two groups: group I and II served as negative and positive controls, the negative control was treated with normal saline while the positive controls received 100mg/kg of the standard drug levamisole. Groups III, IV, V, VI, VII, VIII, IX, X, XI,

and XII were treated with the same two doses of 50mg/kg and 100mg/kg of the crude extract, methanol fraction, n-hexane, ethyl acetate, and Aqueous fractions of the extract. The animals were kept in cages, well ventilated and allowed to have free access to food and water, and also were also allowed to acclimatize for some days before treatment.

Acute toxicity test

Acute toxicity test was done in mice by standard method as described by Lorke (1983) at a dose of 10mg/kg, 100mg/kg, 1000mg/kg, 2600mg/kg, 3900 mg/kg and 5000 mg/kg. A total of 15 mice (15-20 g) were used for this test and they were grouped into groups of three - two for the crude extract per given dose. The first group of three mice received a dose of 10 mg/kg, the second group of three mice received 100 mg/kg, the third group of three mice received 1000mg/kg, the fourth group of two mice received 2600mg/kg, the fifth group of two mice received 3900mg/kg, while the sixth group of two mice received 5000 mg/kg of the crude extract. The animals were then put under observation for twenty (24) hours to check the mortality.

Antigen (Preparation of Sheep red blood cell (SRBC) suspension

Fresh blood was collected from sheep killed in the local slaughterhouse. Sheep red blood cells were washed three times in a large volume of pyrogen-free normal saline and adjusted to a concentration of 1× 10^8cells /ml for immunization and challenge.

Experimental design

A total of sixty (60) Wister albino mice, aged two (2) months and weighing 15-27 g were randomly divided into twelve (12) groups with five (5) mice in each. Group (I) was normal control (NC), group II was Positive control (Levamisole), group III was treated with Crude Extract (50mg/kg), group IV was treated with Crude Extract (100mg/kg), groups V and VI With Ethyl Acetate Fraction, 50 and 100mg/kg respectively, groups VII and VIII were treated with n-hexane Fraction with doses of 50mg/kg and 100mg/kg, groups IX and X were treated with Absolute Methanol Fraction, doses 50mg/kg and 100mg/kg respectively while groups XI and XII was treated with Aqueous Fraction, 50 mg/kg and 100 mg/kg doses respectively

Table 1.1 Summary of different groups of animals and type of treatment administered.

Groups	Treatment type	Dose (mg/kg)
I	Negative control	Normal saline
II	Levamisole	100
III	Crude Extract	50
IV	Crude Extract	100
V	Ethyl Acetate Fraction	50
VI	Ethyl Acetate Fraction	100
VII	n-hexane Fraction	50
VIII	n-hexane Fraction	100
IX	Absolute Methanol Fraction	50
X	Absolute Methanol Fraction	100
XI	Aqueous Fraction	50
XII	Aqueous Fraction	100

Key: NC = Negative Control, EAF = *Ethyl Acetate Fraction*, NF = *n-hexane Fraction*, AMF = *Absolute Methanol Fraction*, AF = Aqueous Fraction.

Infection and treatment of animals

Animals were sensitized on day 0 with 0.1ml of sheep red blood cells, adjusted to a concentration of 1×10^8cells/ml. Treatment of animals was done for 8 days with normal saline, levamisole, crude extract, n-hexane fraction, ethyl acetate fraction, absolute methanol fractions, and aqueous fraction. The concentration for the standard drug levamisole, extract and fractions was 10mg/ml and the doses used for the treatment of mice were 100mg/kg in levamisole with only one group, 50mg/kg and 100mg/kg for crude extract, n-hexane fraction, ethyl acetate fraction, absolute methanol fractions and aqueous fraction with two groups of five mice each.

Invivo immunomodulatory study by Delayed-Type Hypersensitivity Response (DTHR)

Mice were divided into twelve (12) groups of five mice each for the negative control, standard drug levamisole, Crude extract, and four (4) fractions of *Sida acuta* Burm. f. and were immunized on day one by i.p (intraperitoneal) administration of 0.1ml suspension of freshly obtained sheep red blood cells (SRBC) 1×10ˣ8 cells/ml. Experimental groups received orally, calculated volumes of levamisole, crude extract, and its fractions for eight days. On day 8, 2hr after the treatments, animals were exposed to antigens by injecting 0.1ml of sheep red blood cells intradermally in the left hind footpad. The thickness of the foot pads was taken before challenge and at 24hr after challenge. The difference between 0 and 24hrs values of footpad thickness was taken using vernier calipers as a measure of Delayed-type hypersensitivity reaction (The footpad reaction was indicated as the difference in thickness (mm)[8].

$$\text{Percentage paw thickness } (PP_{24}T) = \frac{P_{24} - P_0}{P_0} \times 100\%$$

Where P_{24} and P_o are Paw thickness (mm) PT on the 8th day and 0 day respectively.

$$\text{Percentage paw thickness } (PP_{48}T) = \frac{P_{48} - P_{24}}{P_{24}} \times 100\%$$

Where P_{48} and P_{24} are Paw thickness (mm) PT on the 9th day and 8th day respectively.

Determination of body weight.

The body weights of the animals were determined using a weighing scale during the period of the experiment and percentage body weight changes calculations were done using the following formulae:

$$\text{Percentage body weight change} = \frac{\text{Final body weight} - \text{Initial body weight}}{\text{Initial body weight}} \times 100\%$$

The animals were then sacrificed on the tenth (10th) day using chloroform.

In vitro immunomodulatory activity study by measurement of phagocytosis

To study the effect of the treatment of methanol leave extract of *Sida acuta*, its fractions and levamisole on the activity of polymorphonuclear leucocytes, the *in vitro* slide culture was used [10].

Microorganism

A pure isolate of *Candida albicans* was cultured and isolated in the microbiology laboratory, Department of Microbiology, Faculty of Biological Sciences, University of Nigeria, Nsukka.

Preparation of Candida albicans suspension

According to Ganachari *et. al. "Candida albicans* culture was incubated in Sabouraud dextrose broth overnight and centrifuged to form a cell button at the bottom of the test tube. The supernatant was discarded and the cell button was washed 3-4times with sterile phosphate buffer saline (PBS) and centrifuged. The washed cell button was re-suspended in a mixture of PBS and rat serum in the proportion of 4:1. The count of *Candida albicans* was adjusted to 1×10^8 cells/ml using the 0.5Mc Farland Standard" (2004 P.49)

Preparation of Slide

About 0.2ml of rat blood was smeared on sterile glass slide and incubated at 37^0C for 20mins for clotting to occur. The slide was then drained slowly with sterile normal saline in order not to wash off the attached neutrophils or Polymorphonuclear (PMN) leucocytes. The slides consisting of neutrophils were flooded with the methanol extract of *Sida acuta* Burm.f. and at different concentrations (50, 100 and 200µg/ml), and was incubated 37 ^{o}C for 20mins. The PMN_S was covered with *Candida albicans* suspension and incubated at 37 ^{o}C for 1hr. This was repeated for the standard drug (levamisole) at the concentrations of 50, 100, and 200µg/ml. The slide was drained, fixed with methanol, and stained with Giemsa stain.

Evaluation of Phagocytosis

According to Ganachari: "the slide was observed (100X) under oil immersion and phagocytosis was evaluated by this method, the number of *Candida albican* cells phagocytosed by PMN_S on the slide was determined microscopically for granulocytes using morphological criteria, the number was regarded phagocytic index (PI) and was compared with the PI of the control treatment" (2004, P. 49) Immunostimulation was calculated using the following equation:

$$\text{Percentage Phagocytic Stimulation (PPS)} = \frac{\text{PI (Test)} - \text{PI (Control)}}{\text{PI (Control)}} \times 100$$

Procedure for GC-MS analysis

The GC-MS was done using GCMS-QP2010SE (SHIMADZU), JAPAN instrument. The GC-MS was set up with a capillary column called optima SLB-5MS of length 60m, thickness 0.25µm, ID 0.25mm and a mass selective detector. The electron ionization system was used for detection with ionization energy of 70 eV. Helium was the carrier gas, with a flow rate of 3.22 ml/min. Injector line temperatures was set at 250°C. Column temperature was initially at 60°C, then gradually increased to 260°C at a 3^{o}C /min rate, held for 1.50 min and finally increased to 300°C. Diluted samples (in methanol) of 1.0 µl were injected by split less mode. The components were pin pointed based on the comparison of their relative retention time and mass spectra with those of NIST11 LIB library data of the GC-MS system. The GC-MS gave the different components present in the extracts.

III. RESULTS

The yield of the *Sida acuta* leave extracts was calculated (**Table 1.2**)

Table 1.2: Percentage yield of plant sample

Plant samples	Weight of pulverized samples (g)	Weight of recovered extract (g)	% Yield
**Sida acuta* Burm f.	590	47.18	7.996

Key: *Sida acuta* Burm f.* indicates fresh sample used for extraction

Preliminary phytochemical analysis results

A qualitative phytochemical analysis of the Crude extracts and their fractions showed the presence of alkaloids, flavonoids, saponins, tannins, triterpenoids, steroids and Cardiac glycosides compounds (**Table 1.3**)

Table 1.3: Results of qualitative phytochemical analysis.

Plant Extracts/ Fractions	Phytochemical components						
	Alkaloids	Tannins	Saponins	Flavonoids	Steroids	Cardiac glycosides	Triterpenoids
SrE	+	+	+	+	+	+	-

ISSN: 2349-8889
Volume-8, Issue-5 (September 2021)
www.ijrasb.com
https://doi.org/10.31033/ijrasb.8.5.10

NF	+	+	+	-	+	+	-
EAF	+	+	+	-	+	-	-
AMF	+	-	-	+	-	+	+
AF	+	-	-	-	-	+	+

Key: *— = absent, + = present,* SrE=*Sida acuta Extractl*, NF=n-hexane Fraction, EAF=Ethyl acetate Fraction, AMF=Absolute Methanol Fraction, *and* AF= Aqueous Fraction.

Acute Toxicity Test result

There was no record of mortality in animals, as at the highest dose of 5000mg/kg of the crude extract, no death was observed showing that the crude extract of *Sida acuta* Burm. f. is safe and has a high therapeutic window for use in various *in vivo* pharmacological screening.

Effect of Sida acuta crude extract and fractions of Sida on Delayed-Type Hypersensitivity reaction (DTHR)

Table 1.4: Effect of *Sida acuta* crude extract and fractions on Delayed Type Hypersensitivity reaction (DTHR) in 24 Hours.

S/N	Treatment	Doses (mg/kg)	Paw Thickness (mm)
1.	Control (normal saline)	-	0.300 ± 0.13784
2.	Levamisole	100	0.1800 ± 0.04899
3.	Crude Extract	50	-0.0200 ± 0.04899
		100	0.200 ± 0.07071
4.	Ethyl Acetate Fraction	50	0.2200 ± 0.0800
		100	0.0400 ± 0.0400
5.	n-hexane Fraction	50	-0.02 ± 0.0800
		100	-0.16 ± 0.11225*
6.	Methanol Fraction	50	0.06 ± 0.09274
		100	-0.04 ±0.10770
7.	Aqueous Fraction	50	0.28 ± 0.12410
		100	0.3 ± 0.03162

Values are expressed as mean ± SEM of five observations. ANOVA; Dunnett post hoc test. *P< 0.05 when compared to control.

Table 1.5: Paw Thickness and Percentage Paw thickness of the methanol extract and fractions of Sida acuta Burm.f. leave extract.

S/N	Treatment	PT (mm)	PPT (%)
1.	Control (normal saline)	0.300	13.57
2.	Levamisole	0.1800	6.87
3.	Crude Extract		
	50	-0.0200	0.096
	100	0.200	7.956
4.	Ethyl Acetate Fraction		
	50	0.2200	8.674
	100	0.0400	2.964
5.	n-hexane Fraction		
	50	-0.02	2.656
	100	-0.16	-4.968
6.	Methanol Fraction		
	50	0.06	3.966
	100	-0.04	1.51
7.	Aqueous Fraction		
	50	0.28	11.896
	100	0.3	12.132

Key: **PT = Paw Thickness**
PPT = Percentage Paw Thickness.

Table 1.6: Effect of *Sida acuta* crude extract and fractions on Delayed Type Hypersensitivity reaction (DTHR) in 48hours.

S/N	Treatment	Doses (mg/kg)	Paw Thickness in 48hrs (mm)
1.	Control (normal saline)	-	-0.02 ± 0.03742
2.	Levamisole	100	-0.3 ± 0
3.	Crude Extract	50	-0.1 ± 0.17607
		100	-0.260 ± 0.08718
4.	Ethyl Acetate Fraction	50	-0.206 ± 0.08623
		100	-0.18 ± 0.04899
5.	n-hexane Fraction	50	-0.08± 0.0200
		100	-0.08± 0.05831
6.	Methanol Fraction	50	-0.2 ±0.13416
		100	0.18 ± 0.13191
7.	Aqueous Fraction	50	-0.12± 0.09695
		100	-0.12± 0.03742

Values are expressed as mean ± SEM of five observations. There is no significant difference in paw thickness reduction after 48hours P > 0.05

Table 1.7: Paw Thickness and Percentage Paw thickness of the methanol extract and fractions of *Sida acuta Burm.f. leave extract in* 48hrs.

S/N	Treatment	PT in 48hrs (mm)	PPT (%)
1.	Control (normal saline)	-0.02	-0.664
2.	Levamisole	-0.3	-10.284
3.	Crude Extract		
	50	-0.1	-6.544
	100	-0.26	-9.198
4.	Ethyl Acetate Fraction		
	50	-0.206	-7.082
	100	-0.18	-6.426
5.	n-hexane Fraction		
	50	-0.08	-1.428
	100	-0.08	-2.008
6.	Methanol Fraction		
	50	-0.2	-9.824
	100	-0.18	-6.048
7.	Aqueous Fraction		
	50	-0.12	-3.924
	100	-0.12	-4.338

Key: PT = Paw Thickness
PPT = Percentage Paw Thickness.

Table 1.8: Paw Thickness comparison of the methanol extract and fractions of *Sida acuta* Burm. f. *leave extract in* 24hours and 48hours.

S/N	Treatment (mg/kg)	PT in 24hrs (mm)	PT in 48hrs (mm)
1.	Control (normal saline)	0.3	-0.02
2.	Levamisole	0.18	-0.3
3.	Crude Extract		
	50	-0.02	-0.1
	100	0.2	-0.26
4.	Ethyl Acetate Fraction		
	50	0.22	-0.206
	100	0.04	-0.18
5.	n-hexane Fraction		
	50	-0.02	-0.08
	100	-0.16	-0.08
6.	Methanol Fraction		
	50	0.06	-0.2

		100	-0.04	-0.18
7.	Aqueous Fraction			
		50	0.28	-0.12
		100	0.3	-0.12

Key: PT = Paw Thickness

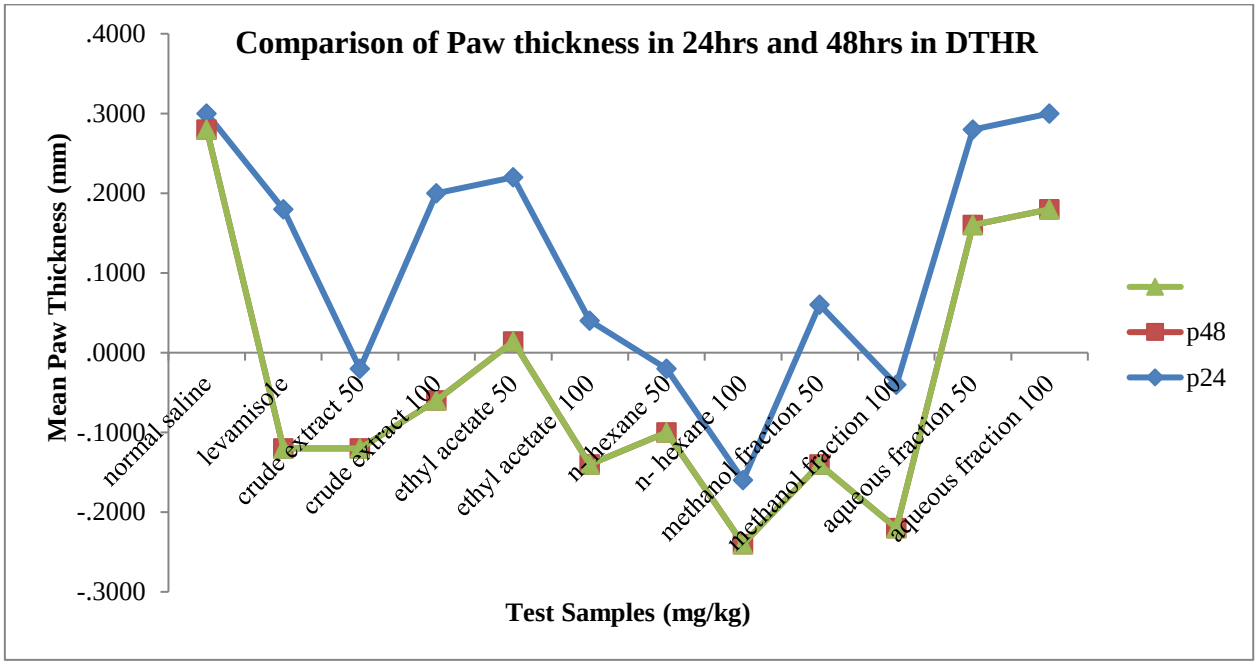

Fig 1.2: Paw thickness comparison in 24hours and 48hours of the methanol extract of *Sida acuta* crude Extract (SrE), Levamisole (Leva), n-hexane fraction (NF), Ethyl Acetate fraction (EAF), Aqueous fraction (AF), Absolute Methanol fraction (AMF) and normal saline (negative control). There is an immune response in the body of the activated mice at 24hours which acts against the antigen to decrease inflammation of the left paw of the mice towards the 48hours.

Table 1.9: Effect of *Sida acuta* crude extract and fractions on body weight of mice in Delayed-Type Hypersensitivity reaction (DTHR).

S/N	Treatment	Doses (mg/kg)	Body weight Change (g)
1.	Control (normal saline)	-	1.79 ± 0.49953
2.	Levamisole	100	1.174 ± 0.03919
3.	Crude Extract	50 100	2.048 ± 0.83208 1.238 ± 0.8469
4.	Ethyl Acetate Fraction	50 100	1.928 ± 0.55331 0.38 ± 1.34389
5.	n-hexane Fraction	50 100	2.356± 0.48728 0.632 ± 1.0181
6.	Methanol Fraction	50 100	0.262 ± 0.96874 2.646 ± 0.57007
7.	Aqueous Fraction	50 100	0.83 ± 0.86353 2.016 ± 0.82476

Values are expressed as mean ± SEM of five observations. There is no significant difference in body weight of mice P > 0.05.

ISSN: 2349-8889
Volume-8, Issue-5 (September 2021)

www.ijrasb.com https://doi.org/10.31033/ijrasb.8.5.10

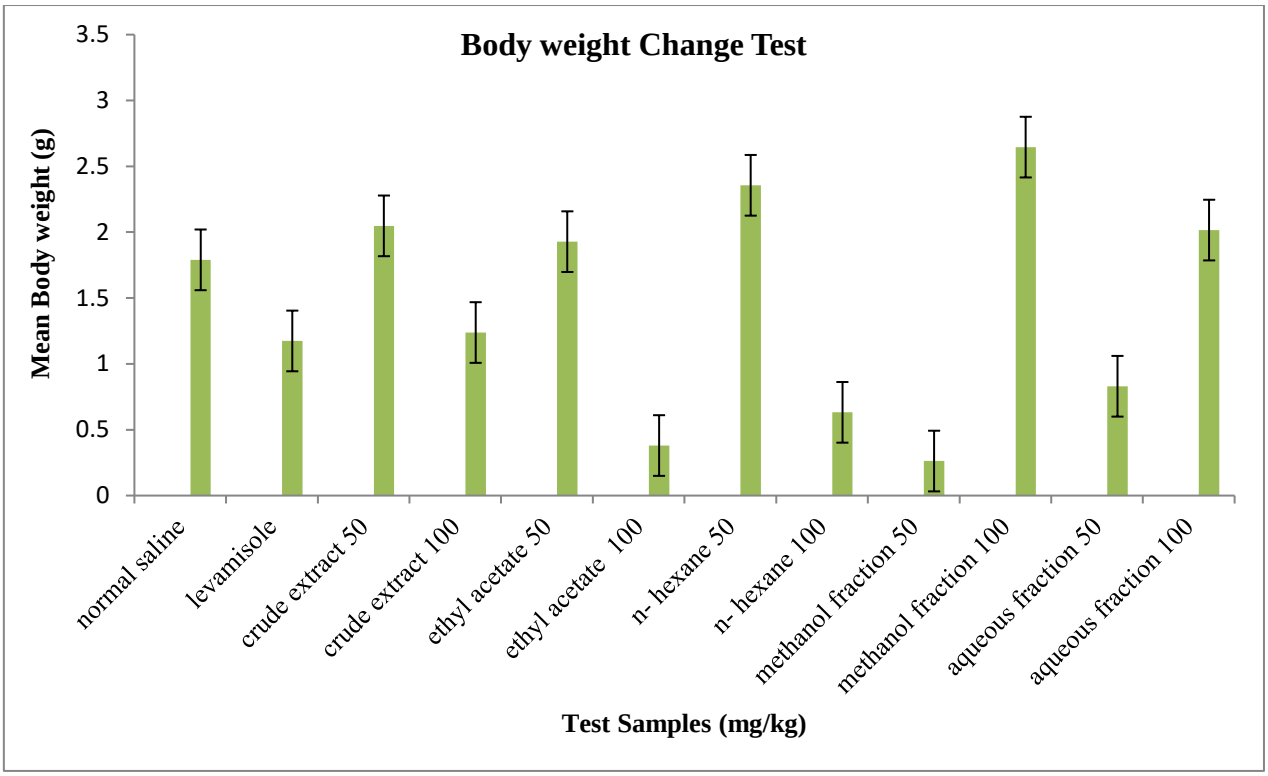

Figure 1.3: Body weight change of the methanol extract of *Sida acuta crude Extract* (SrE), Levamisole (Leva), n-hexane fraction (NF), Ethyl Acetate fraction (EAF), Aqueous fraction (AF), Absolute Methanol fraction (AMF) and normal saline (negative control).

Table 1.10: Body weight Change and Percentage Body weight Change of the methanol extract and fractions of *Sida acuta* Burm. f. *leave extract in* Delayed-Type Hypersensitivity reaction.

S/N	Treatment	BWC (g)	PBWC (%)
1.	Control (normal saline)	1.79	13.928
2.	Levamisole	1.174	7.096
3.	Crude Extract		
	50	2.048	10.272
	100	1.238	8.504
4.	Ethyl Acetate Fraction		
	50	1.928	11.454
	100	0.38	-4.326
5.	n-hexane Fraction		
	50	2.356	11.988
	100	0.632	3.894
6.	Methanol Fraction		
	50	0.262	-0.492
	100	2.646	19.706
7.	Aqueous Fraction		
	50	0.83	6.914
	100	2.016	16.986

Key: BWC = Body Weight Change
PBWC = Percentage Body weight Change

Effect of methanol crude extract and fractions of Sida acuta on phagocytosis of Candida albicans

Table 1.11: Phagocytic activity of polymorphonuclear leucocytes.

Treatment	Concentration(µg/ml)	Phagocytic index (PI)
Control (normal saline)	-	31 ± 2.333
Levamisole	50	217 ± 16.666*
	100	383 ± 16.666*
	200	400 ± 0*
SrE	50	177 ± 14.530*
	100	277 ± 14.530*
	200	300 ± 0*
NF	50	99±25.569*
	100	103± 9.451*
	200	173 ± 14.530*
EAF	50	44 ± 0.332
	100	73 ± 1.666
	200	100 ± 0*
AF	50	65 ± 10.408
	100	83 ± 24.037
	200	63 ± 12.018
AMF	50	49 ± 2.333
	100	87 ± 9.279*
	200	82 ± 10.138

Values are expressed as mean ± SEM of three observations. ANOVA; Dunnett post hoc test. *P< 0.05 when compared to control.

Key: — = *absent*, + = *present*, SrE=*Sida acuta Extractl*, NF=n-hexane Fraction, EAF=Ethyl acetate Fraction, AMF=Absolute Methanol Fraction, *and* AF= Aqueous Fraction.

Table 1.12: Phagocytic Index and Percentage Phagocytic Stimulation of the methanol extract of *Sida acuta* Burm. f. leaves, its fractions and levamisole.

Treatment	PI	PPS (%)
Control (normal saline)	31± 2.33	-
Levamisole		
50µg/ml	217±16.666	600
100µg/ml	383±16.666	1135.48
200µg/ml	400±0	1190.32
SrE		
50µg/ml	177±14.530	470.96
100µg/ml	277±14.530	793.54
200µg /ml	300±0	867.74
NF		
50µg/ml	99±25.569	219.35
100µg/ml	103±9.451	232.25
200µg/ml	173±14.530	458.06
EAF		
50µ/ml	44±0.332	41.93
100µ/ml	73±1.666	135.48
200µ/ml	100±0	222.58
AF		
50µ/ml	65±10.408	109.67
100µ/ml	83±24.037	167.74
200µ/ml	63±12.018	103.22

AMF		
50µ/ml	49±2.333	58.06
100µ/ml	87±9.279	180.64
200µ/ml	82±10.138	164.51

Key: PI = Phagocytic Index
PPS = Percentage Phagocytic Stimulation

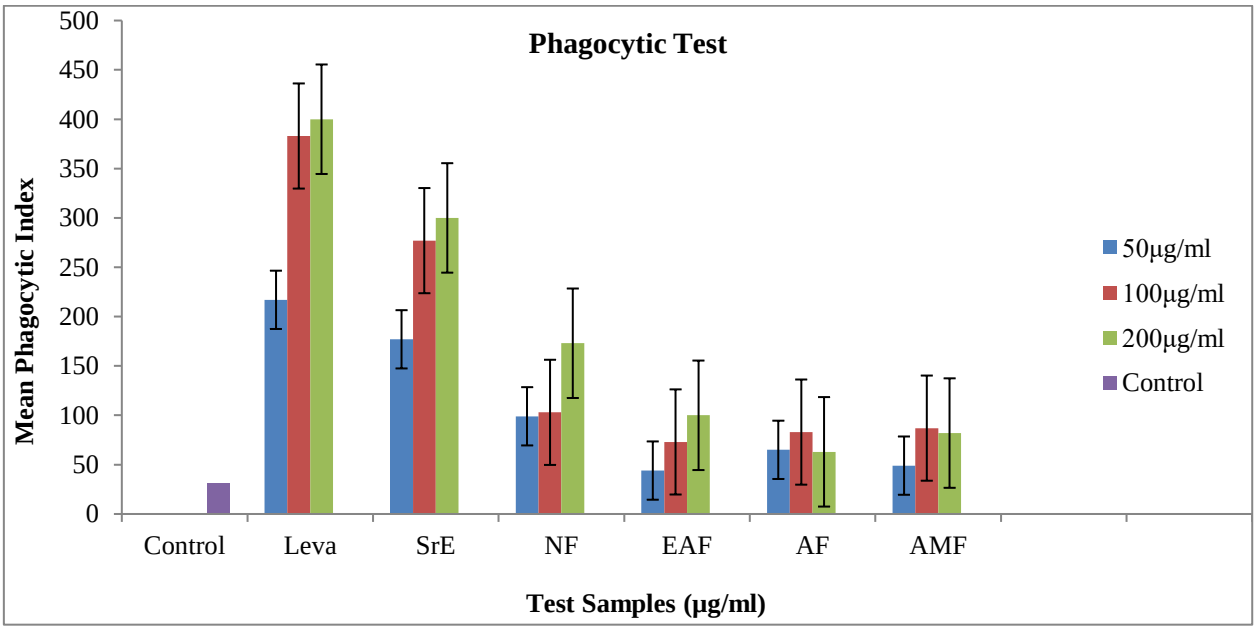

Fig 1.4: Phagocytic Indexes (PI) of methanol extract and fractions of *Sida acuta* PI showed the degree of phagocytic activity of polymorphonuclear leucocytes in various test samples and at different concentrations.

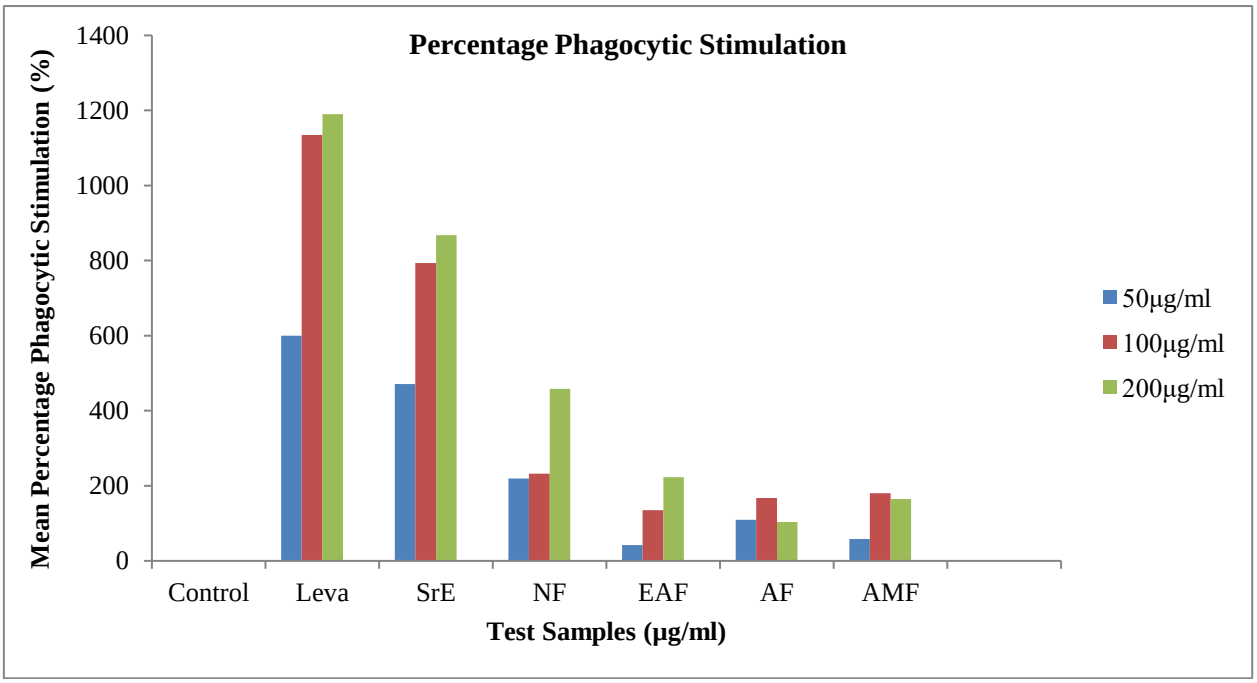

Fig 1.5: Percentage of phagocytic stimulation of the methanol extract and fractions of *Sida acuta*

GC-MS SPECTRA OF n-HEXANE FRACTION
GCMS-QP2010SE SHIMADZU, JAPAN

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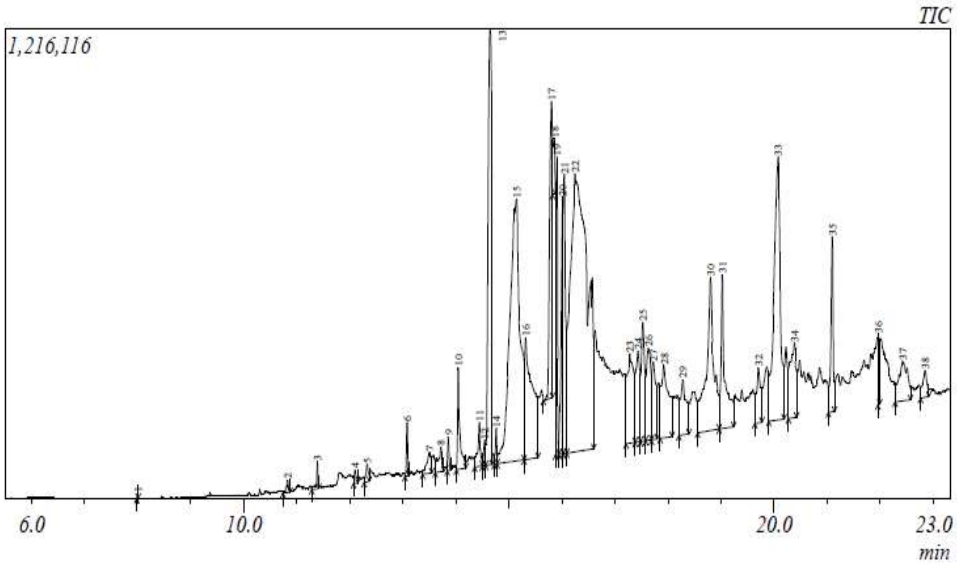

Figure 1.6: GC-MS spectrum for n-hexane fraction of *Sida acuta Burm.*

Results of GC-MS analysis of n-hexane fraction.

Table 1.13: GC-MS analysis of n-hexane fraction of *Sida acuta Burm.f.*

Peak number	RT	% PA	M_w	Molecular formula	Name of compound	Biological activity
6.	13.080	0.31	296	$C_{19}H_{36}O_2$	Cyclopentane tridecanoic acid, Methyl ester.	Antimicrobial [19]
10.	14.045	1.12	268	$C_{18}H_{36}O$	2-Pentadecanone,6,10,14-trimethyl	Anti-inflammatory [11]
13.	14.644	6.18	270	$C_{17}H_{34}O_2$	13 Hexadecanoic acid, methyl ester.	Anti- inflammatory [29]
15.	15.145	12.57	256	$C_{16}H_{32}O_2$	n-Hexadecanoic acid	Anti-inflammatory [30]
17.	15.800	4.19	294	$C_{19}H_{34}O_2$	9,12-octadecadienoicacid methyl ester.	Antifungal [15]
21.	16.046	2.77	298	$C_{19}H_{38}O_2$	Methyl stearate	Antioxidant [32].
22.	16.250	22.20	280	$C_{18}H_{32}O_2$	9,12-octadecadienoic acid (Z,Z)	Antifungal [24].
25.	17.524	1.76	374	$C_{25}H_{42}O_2$	25- Cyclopropane butanoic acid2-[[2-[[2-[(2-pentylcyclopropyl)methyl]cyclopropyl] Methyl]-,methyl ester.	Antioxidant [31]
30.	18.799	5.40	330	$C_{19}H_{38}O_4$	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester.	Antiplasmodial [3]
33.	20.073	7.99	354	$C_{21}H_{38}O_4$	9,12-Octadecadienoic acid (Z,Z)-2,3-dihydroxypropylester.	Anti-inflammatory, Anticancer [30].
36.	21.958	0.60	638	$C_{39}H_{74}O_6$	Dodecanoic acid,1,2,3-propanetriylester ester.	Hepatoprotection [1]
38.	22.843	0.53	416	$C_{28}H_{48}O_2$	3,4-seco-5alpha-cholestan-3-oic acid,4-hydroxy-4-methyl-epsilon-lactone, (4R).	Not reported

PA= Peak Area, RT=retention time, RI=retention index, M_w=Molecular weight

Result of Statistical Analysis

The results obtained from the experiment were expressed as mean ±SEM and they were analyzed using one-way analysis of variance (one-way ANOVA) to determine the significance level ($p<0.05$) followed by Dunnett Post hoc test. The SPSS (version 20) was used for the analysis.

IV. DISCUSSION

The *sida acuta* extraction yield showed a less yield of approximately 8% which is in line with the work of Alagbe and Shittu 2020. The result for the phytochemical composition shows the presence of flavonoids, tannins, alkaloids, steroids, triterpenoids,

cardiac glycosides, and saponins, which are responsible for the immunomodulatory activity of this plant extract and that was in agreement with earlier work done by [22] and [4]. The different secondary metabolites possessed by the plant have led to its increased pharmacological activities like anti-inflammatory, antioxidative, hypoglycaemic, antibacterial, immunomodulatory activities. [25]. Flavonoids are known for their antioxidative activity, tannins possess antibacterial, antiviral, and potent against most destructive diseases [21]. Steroids are antifungal, hypotensive, and hypocholesterolemic [17]. Glycosides are a potent signal transducer and have several intracellular targets, thus modulating the activities of enzymes and hormones [20]. The result of the acute toxicity test revealed that the extract has a very wide safety margin and is safe for the mice and that was in agreement with work done by [2]. The LD_{50} of the methanol leave extract was greater than 5000mg/kg since there was no death or any sign of toxicity after 24hrs observations according to lorkes, and the oral LD50 of the extract was then taken to be > 5000mg/kg which is similar to work done by [23]. The presence of alkaloids showed potent antimicrobial activity against several other microorganisms [13] which is in line with the *in vitro* phagocytosis of the Polymorphonuclear leukocytes (PMNs) cells activated with the plant extract and fractions on *candida albicans*, it evoked 867.74% of phagocytic stimulation at 200µg/ml concentration *in vitro* phagocytic study. Also, the n-hexane fraction significantly ($p < 0.05$) showed dose-related stimulation of cellular immunity at the higher percentage of 458.06% at 200µg/ml. Aqueous fraction showed a non-significant ($P > 0.05$) phagocytic index while the standard drug Levamisole exhibited a significant ($p < 0.05$) dose-related Percentage Phagocytic Stimulation with the highest Percentage Phagocytic Stimulation of 1190.32% at 200µg/ml. The Absolute methanol fraction showed a high PPS of 180.64% at 100µg/ml, Ethyl Acetate Fraction at 222.58% at 200µg/ml and also showed a statistically significant difference ($P < 0.05$) when compared to the control group. The *in vivo* Delay Type Hypersensitivity Reaction DTHR is a vital immune system reaction in an organism.it assesses the potency of plant extracts on cell-mediated immune actions, especially in immunomodulatory screening by measuring the paw thickness after the tested animals were injected with the antigen [28]. The *Sida acuta* leave extract exhibited 7.596% of the DTHR at 100mg/kg dose.

The increase in DTHR to sheep red blood cells shows the activation of adaptive immune system, which is a key player in the cure of infections. The reaction is initiated by the detection and activation of T-lymphocytes before the main challenge with a known antigen that was Sheep Red Blood Cells, SRBC. The activation of DTHR results in multiplication and release of cytokines by T- lymphocytes which cause an increase in vascular permeability, induces vasodilation, activates, and builds up macrophage cells. This improves phagocytosis that's kills the antigen [26]. The term delayed is used to differentiate a secondary cellular response which appears 24- 72hrs from the immediate hypersensitivity response that appears within 12mins of an antigen challenge. The DTHR in paw thickness shows the value of immune reaction. It is reflected by the sum values of the number of responses, a positive DTHR response is shown by foot pad swelling in response to donor antigen, while the negative response is characterized by no footpad swelling and maybe as a result of regulation or deletional tolerance. The DTHR is characterized by an increase in the influx of non-specific inflammatory cells, having macrophage as a special component. It is a type IV hypersensitivity that manifests when antigen activates sensitized T cells. T-cells activation results in the secretion of different cytokines including interleukin-2, interferon-γ, macrophage migration inhibition factor, and tumor necrosis factor – β [6].

The T-cell mediated DTH response to sheep red blood cells showed a low in paw volume in test groups when compared to the control group, amongst the test groups, n-hexane fraction showed an intense decrease in paw volume which is very much significant when compared to the control groups. The percentage decrease in the formation of edema in the n-hexane fraction treated group is -4.968% at 100mg/ml and -2.008% within 24hours and 48hours respectively. There is no significant change in the body weight change of mice in various treated groups when compared to control groups ($P > 0.05$) and also no significant change ($P > 0.05$) in paw thickness reduction within 48 hours of DTH response. There is a corresponding increase in the weight of the mice which does not have a significant difference ($P > 0.05$) when compared to the control groups. The Gas Chromatography coupled Mass spectrometry (GC-MS) is a technical approach for pin-pointing active bio-compounds in medicinal herbs which includes unstable materials, long and branched chain hydrocarbons, alcohols, acid esters, etc. for quantitative analysis, gas chromatography coupled flame ionization detector (GC-FID) and GC-MS are preferred [5]. More so, the mass spectra of these compounds were analysed with those stored in the NST11 library and the results were shown in table 1.13. GC-MS analysis of fraction with highest activity was carried out on n-hexane fraction which showed the presence of some compounds like hexadecanoic acid, 2-hydroxy-1 (hydroxymethyl) ethyl ester, 3,4-seco-5alpha-cholestan-3-oic acid,4-hydroxy-4-methyl epsilon-lactone, (4R), 2- pentadecanone,6, 10, 14-trimethyl, 9,12-octadecadienoic acid, methyl ester, methyl stearate, cyclopentane tridecanoic acid, methyl ester, cyclopropane butanoic acid, 2-[[2-[[2-[(2-pentylcyclopropyl) methyl]cyclopropyl] methyl] methyl ester etc. and the presence of these bioactive compounds is a proof that *sida acuta* Burm. f. leaves can stimulate a

depressed immune system, hence acting as a potent immunomodulant.

V. CONCLUSION

The result of the study indicated that methanol leave extract of *Sida acuta* Burm. f. leaves possess immunomodulatory action with significant effect in its fractions and can help in modulating an immune compromised system as well as immune disorders. The extract has a very wide safety margin and was able to boost immune response which acted on the antigens and lead to reduction in the paw size of the infected mice and the phagocytic test showed a very significant difference in the phagocytic activity of the polymorphonuclear neutrophils in the extract and its fractions when compared with the control group. The phytochemical analysis revealed the presence of flavonoids, saponins, alkaloids, triterpenoids, cardiac glycosides, steroids and tannins, while that of GC-MS showed the presence of compounds like hexadecenoic acid, 2-hydroxy-1 (hydroxymethyl) ethyl ester, 3,4-seco-5alpha-cholestan-3-oic acid,4-hydroxy-4-methyl epsilon-lactone, (4R), 2-pentadecanone,6, 10, 14-trimethyl, 9,12-octadecadienoic acid, methyl ester, methyl stearate, cyclopentane tridecanoic acid, methyl ester, cyclopropane butanoic acid, 2-[[2-[[2-[(2-pentylcyclopropyl) methyl]cyclopropyl] methyl] methyl ester etc. which have reports that showed their immunomodulatory properties and indicates that the extract and fractions of *Sida acuta* Burm. f. leaves are a promising immunomodulatory drug candidate.

RECOMMENDATIONS

- This research has established the immunomodulatory activity of methanol leave extract of *Sida acuta* Burm. f. and its fractions. However, further work could still be done on this project and we therefore recommend that;
- The immunomodulatory activity should be carried out in other *invitro* models.
- Other *invivo* studies should be carried out with increased doses of the extract and fractions.
- Histopathological and hematological studies could also be carried out in further research to check the effect of the extracts and its fractions on some organs such as the liver, kidney, heart and pancreas.

ACKNOWLEDGEMENT

The authors are thankful to Mr. and Mrs. Alaefuna, Martin N. for helping to fund this project and to Prof. Anyanwu, Chukwudi U., Technologist Ugboaja, Oluchi, Prof. Ebi, G., Dr. Okpala, C.O., Dr. Eze, F. I., Pharm. Adaka, C. I., Mr. Nurudeen Ade, and Dr. Wilfred Ugwuoke for their assistance.

CONFLICT OF INTEREST

None

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