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ORIGINAL RESEARCH

In-vivo Immunomodulatory Activities of Essential Oils of *Artemisia abyssinica* and *Lepidium sativum* in Mice

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Background: Ethiopians use *Artemisia abyssinica and Lepidium sativum* as immunity enhancers. However, there is no scientific validation conducted so far regarding this claim. The aim of this study was to investigate the in-vivo immunomodulatory activities of essential oils of *A. abyssinica and L. sativum* in mice.

Methods: The extraction was carried out using the earlier techniques. By hydro distilling fresh seeds and aerial portions of *A. abyssinica* and *L. sativum*, respectively, essential oils were obtained. Essential oils of both plants were tested at 100, 200 and 400 mg/kg. The rate of carbon clearance, humoral antibody titer, delayed type hypersensitivity response, spleen and thymus indices were evaluated in mice according to scientific protocols. The carbon clearance assay was determined using carbon ink. Sheep red blood cell was used as an antigen for other tests.

Results: Essential oils of *A. abyssinica* and *L. sativum* at 400 mg/kg significantly increased the rate of carbon clearance from the body of mice ($p<0.05$). The maximum carbon clearance rate was achieved for *A. byssinica* essential oil at 400 mg/kg. Both essential oils raised the level of HAT to SRBC in comparison to the vehicle and cyclophosphamide administered groups. The largest (84.668±1.951) mean secondary HAT to SRBC was generated by *L. sativum* essential oil at 400 mg/kg ($p<0.001$). *A. abyssinica* essential oil at 200 and 400 mg/kg significantly increased the level of thymus index compared to the model group ($p<0.05$ and 0.01 respectively). The levamisole group experienced the highest increase in thymus index ($p<0.001$). Essential oil of *L. sativum* at 400 mg/kg also increased the level of thymus index. The spleen index in mice was improved by the essential oils only at the highest dose levels (400 mg/kg).

Conclusion: It can be inferred that the essential oils of *L. sativum* and *A. abyssinica* have immunostimulant properties.

Keywords: *Artemisia abyssinica*, *Lepidium sativum*, immunomodulatory, in-vivo

Introduction

When the immune system's components are hampered, health problems can develop.[1] Infectious diseases are more likely to affect patients with compromised immune systems.[2] Others include arthritis, ulcerative colitis, asthma, allergies, and cancer.[3] The immune system can be stimulated or suppressed by immunomodulators.[4]

Recently, different synthetic and natural immunomodulatory medicines have been developed. Most of these are cytotoxic, have immunosuppressive activity and a variety of side effects. Investigations into medicinal plants and chemicals derived that can alter particular immune responses are the focus of researchers. Immunostimulatory medicinal herbs showed reduced toxicity and adverse consequences.[5] *Artemisia abyssinica* and *Lepidium sativum* are used in Ethiopian for a variety of therapeutic properties.[6,7]

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The *Asteraceae* plant, *A. abyssinica*, is named as "chikugn" in Ethiopia. It is a perennial or annual herb that grows straight and 30 to 60 cm tall. The plant is used regularly in both traditional medicine and rituals. It has been used as a treatment for leprosy, gonorrhea, tonsillitis, rabies, cough, and syphilis in human. The freshly harvested roots are used to treat epileptic animals.[8,9] The anthelmintic, antibacterial, antispasmodic, and antirheumatic activities were reported in conventional medicine. Additionally, antioxidant, antileishmanial, and antitrypanosomal activities of *A. abyssinica* essential oil have been discovered.[10,11] Previous phytochemical analyses reported the presence of 4, 5-dihydroxyocta-3, 5-diene-2, 7-dione (55.0%), yomogi alcohol (38.5%), artemisyl acetate (24.9%), 4-hydroxycyclohexanemethanol (21.3%) and -terpinolene (9.2%) as well as artemisia alcohol (6.7%).[12]

L. sativum is a member of the *Brassicaceae* family.[13] In English, it is referred to as "Garden cress". In Ethiopia, it is known as "fetto". L. sativum is frequently grown as a garden plant in addition to being sold in any market.[14] Though seeds, leaves, and roots are commercially valuable, L. *sativum* is mostly planted for seeds.[15,16] The seed oils are used to treat clinical problems, including migraine, arthritic pain, hypertension, hyperglycemia, hepatitis, menstrual irregularities, and erectile dysfunction, fractures, diarrhea, and vitamin C deficiency.[17,18] Moreover, it has been connected to pharmacological properties as galactagogue, immune system enhancer, anticancer, antioxidant, laxative, febrifuge, and diuretic effects.[19,20] The antioxidant activity of seed oil extract is superior to leaf oil extract, according to a recent study. However, the leaf oil possesses better antibacterial activity.[20,21] Flavonoids, phenolics, alkaloids, saponins, glycosides, coumarins, fixed oils, proteins, vitamins, and minerals were found in *L. sativum* essential oil.[22]

The antioxidant qualities of the active components of *A. abyssinica* and *L. sativum*, which are responsible for immunomodulatory capabilities, may explain their traditional applications.[23] In this study, the essential oils of *A. abyssinica* and *L. sativum* were tested in mice to see how they modulated immune systems.

Materials and Methods

Chemicals, Reagents and Drugs

This study utilized analytical-grade substances such as cyclophosphamide (Cadila Pharmaceuticals Ltd., India), carbon ink (Ankur Minerals Pvt. Ltd., India), and levamisole (Xingtai Yuanao Technology Co., Ltd., China).

Plant Material

L. sativum seeds and aerial portions of *A. abyssinica* were collected in Debre Berhan, North Shewa Zone, Amhara National Regional State, Ethiopia. The plants' veracity was confirmed by the herbarium of the biology department of Addis Ababa University in Ethiopia. The voucher specimens for *A. abyssinica* and *L. sativum* were given the reference numbers AA/2023 and LS/2023, respectively.

Experimental Animals

Swiss albino male mice, weighing 25–35 g, were used. They were 2–3 months old. Mice were obtained from the Ethiopian Public Health Institute in Addis Ababa, Ethiopia. They feed standard pellet and tape water ad libitum. The animals were acclimatized to the testing area for five days before the experiment. Guidelines for the management and use of experimental animals were followed.[24]

Preparation of Essential Oils

By hydro distilling fresh seeds and aerial portions of *A. abyssinica* and *L. sativum*, respectively, essential oils were produced. For extraction, a device of the Clevenger type was used. 1000 g of each of the fresh plant materials underwent an 8-hour distillation process. The procedure was carried out using the earlier techniques.[25]

Sheep Red Blood Cell Preparation (SRBC)

Blood was collected from the external jugular vein of a sheep. It was mixed 1:1 with freshly prepared Alsever's solution. SRBCs were extracted from the collected blood by centrifuging at 2500 revolutions per minute (rpm) for 10 minutes. The extract was washed in pyrogen free normal saline (0.9% w/v). In 1 mL of sheep blood, 1×10^8 SRBC was adjusted to be found.[26,27]

Preparation of Carbon Ink Suspension

Carbon ink was diluted with eight volumes of ordinary saline and used as an antigen for carbon clearance assay.[28]

Vehicle and Standard Drug Selection

Essential oils are not water soluble, whereas Tween 80 has immunomodulatory effects.[29] The 2% concentration of gum acacia was used as the suspending agent. Levamisole can cause immunological reactions on the cellular and humoral levels. It is effective immunostimulant.[30] Cyclophosphamide is a common immunosuppressant. It has antimitotic effects and may also cause inhibition of the immune system's T-cell-mediated response.[31]

Animal Grouping and Dosing for Carbon Clearance Test and Humoral Antibody Titer Response

The median lethal dose (LD50) of the essential oils of *A. abyssinica* and *L. sativum* was calculated by an acute toxicity test. The LD50 of both essential oils was more than 2000 mg/kg. The selected treatment doses were 100, 200, and 400 mg/kg.[32] Mice were randomly grouped into nine groups of five mice each. Group 1 received the vehicle at 10 mL/kg. Levamisole and cyclophosphamide were administered to groups II and III at doses of 50 and 30 mg/kg, respectively. *A. abyssinica* essential oils were administered in doses of 100, 200, and 400 mg/kg to Groups IV–VI, respectively. The last groups (VII–X) were treated with L. *sativum* essential oil of 100, 200, and 400 mg/kg, respectively. In this experiment, a total of 90 mice were employed.

Carbon Clearance Test

The test was conducted using methods that have been documented.[27,33] According to the aforementioned protocol, mice were allocated into nine groups and received the proper treatments for five days. 48 hours following the previous dose, all groups received 10mL/kg of carbon ink suspension intravenously through the tail vein. Blood samples were obtained 5 and 15 minutes after injecting the solution into mice. Using a 675 nm absorbance test, the 25 µL of collected blood was mixed with 3 mL of 0.1% sodium carbonate to determine the optical density. The rate of carbon removal from optical densities was determined using Equation 1.

$$k = \frac{\ln OD1 - \ln OD2}{(t2 - t1)} \quad (1)$$

Where, K: carbon clearance rate.

OD1: Optical density at a time (t1), 5 min after ink injection.

OD2: Optical density at a time (t2), 15 min after ink injection.

Humoral Antibody Titer (HAT) Response to SRBC

The scientific methods were considered to evaluate the humoral response to SRBC.[34,35] Each mouse received an intraperitoneal injection of 0.1 mL of SRBC suspension containing 1×10^8 cells/mL. Mice were then randomly distributed into nine groups according to the approach previously described. The 14-days course of treatment started 24 hours after the vaccination. On day 7, an hour following the administration of the dose, all mouse groups were challenged with an intraperitoneal (i.p.) injection of 0.1 mL of SRBC solution in normal saline containing 1×10^8 cells /mL. On the fourteenth day, blood was drawn from each mouse's retro orbital plexus and centrifuged for 10 minutes at 2500 rpm to separate the serum. The % change in HAT compared to the control group was calculated using Equation 2.

$$\%\ \text{Change} = \frac{(\text{HAT treatment} - \text{HAT control}) \times 100}{\text{HAT treatment}} \quad (2)$$

Determination of Delayed Type Hypersensitivity (DTH)

DTH was determined by footpad swelling test. Mice grouping and experimental designs were as follows. Mice were randomly divided into nine groups (n=5): normal group, model group, 6 test groups and positive treatment group. Mice

from the normal group were administered with the vehicle at 10 mL/kg. Levamisole and cyclophosphamide were given to Groups II and III at doses of 50 and 30 mg/kg and considered as positive treatment and model groups, respectively. Groups IV–VI were treated with *A. abyssinica* essential oils at 100, 200, and 400 mg/kg, respectively. The last test groups (VII–X) received doses of 100, 200, and 400 mg/kg of *L. sativum* essential oil, respectively. All respective treatments were provided for seven days. On day 2, 4 and 6, cyclophosphamide (30 mg/kg) was administered to all test and positive treatment groups. Mice were sensitized on day 2 by administering 0.2 mL of 2% SRBC i.p. They were also subcutaneously injected with 20 µL of 20% SRBC on day 7. After 24 hours, the left and right rear footpads' thicknesses were measured. The difference in thickness between the left and right rear footpads before and after the attack was used as a proxy for the degree of DTH.[36]

Determination of Immune Organ Index

The experimental setup and mouse grouping were the same as delayed type hypersensitivity test. Animals were weighed and put to death using sodium pentobarbital anesthesia on day 7. The spleen and thymus were removed and weighed. The weight of the thymus or spleen/body weight formula was used to compute the thymus and spleen indices.[36]

Data Reliability

Each test was run twice to ensure that the results were reproducible. Data obtained in the second phase of the experiment was taken into consideration for analysis since it demonstrated a high degree of reliability with the first phase.

Ethical Consideration

Before the study began, the Institutional Review Board of Debre Berhan University in Ethiopia gave its approval. A letter of clearance was granted. Additionally, every precaution was made to prevent animal suffering during the trial. All methods were carried out in accordance with relevant guidelines and regulations. All methods were reported considering ARRIVE guidelines.

Data Analysis

The statistical package for social science (SPSS) version 21 software was used to analyses the data, which were presented as Mean ± SEM (Standard Error of Mean). One-way ANOVA was used to compare the statistical differences between the groups, and then the Tukey post hoc multiple comparison test. When the p-value at a 95% confidence interval was less than 0.05, the results were deemed significant.

Results

As shown in Table 1, mice treated with *A. abyssinica* essential oils resulted in greater clearance of carbon particles than vehicle or cyclophosphamide treated groups. Similarly, when compared to the vehicle and cyclophosphamide groups, mice administered with *L. sativum* essential oil showed a higher rate of carbon elimination. The highest doses had a more noticeable effect. When compared with vehicle, the essential oils of *A. abyssinica* and *L. sativum* at 400 mg/kg significantly increased the rate at which carbon was cleared from the body of mice ($p<0.05$).

The maximum carbon clearance rate was achieved when *A. byssinica* essential oil at 400 mg/kg was used in comparison to *L. sativum* and levamisole treated groups. A 400 mg/kg dose of *L. sativum* essential oil stimulated carbon removal similarly to levamisole. *L. sativum* essential oil had a better outcome on mice's carbon clearance at lower doses compared to *A. abyssinica* essential oil of comparable doses (Figure 1). In comparison to mice given the vehicle and cyclophosphamide treated groups, both essential oils at lower doses (100 and 200 mg/kg) showed a statistically negligible increase in the mean carbon clearance rate.

Table 2 shows that *A. abyssinica* and *L. sativum* essential oils raised the level of HAT to SRBC in comparison to the vehicle and cyclophosphamide administered groups. The largest (84.668±1.951) statistically significant ($p<0.001$) mean secondary HAT to SRBC was generated by *L. sativum* essential oil at 400 mg/kg, increasing the level of HAT by 61.23%. At 400 mg/kg, the *A. abyssinica* essential oil raised the level of secondary HAT to SRBC by 59.87% when compared to the vehicle.

Table 1 The Effect of *A. abyssinica* and *L. sativum* Essential Oils on Mice's Carbon Clearance Rate (CCR)

Groups	Dose (mg/kg)	CCR (Mean ± SER)	95% Confidence Interval	
			Lower Bound	Upper Bound
Vehicle	——	0.050±.020	0.010	0.090
Levamisole	50	$0.172\pm.020^{V**C***A1*}$	0.132	0.212
Cyclophosphamide	30	0.033±.020	−0.007	0.073
LSEC1	100	$0.084\pm.020^{A4**}$	0.044	0.124
LSEC2	200	0.119±.020	0.079	0.159
LSEC4	400	$0.166\pm.020^{V**}$	0.126	0.206
AAEC1	100	$0.066\pm.020^{L*LS4*A4**}$	0.026	0.106
AAEC2	200	$0.108\pm.020^{A4*}$	0.068	0.148
AAEC4	400	$0.200\pm.020^{V***C***LS1**A1**A2*}$	0.160	0.240

Notes: Results are expressed as mean ± SEM; n=5. ^{V}Compared to the vehicle, ^{C}Compared to cyclophosphamide, ^{A1}Compared to *A. abyssinica* essential oil at 100 mg/kg, ^{A2}Compared to *A. abyssinica* essential oil at 200 mg/kg, ^{A4}Compared to *A. abyssinica* essential oil at 400 mg/kg, ^{L}Compared to levamisole, ^{LS1}Compared to *L. sativum* essential oil at 100 mg /kg and ^{LS4}Compared to *L. sativum* essential oil at 400 mg /kg. *P<0.05, **P<0.01, ***P<0.001.

Abbreviations: SEM, Standard error of the mean; LSEC1, *L. sativum* essential oil at 100 mg /kg; LSEC2, *L. sativum* essential oil at 200 mg /kg; LSEC4, *L. sativum* essential oil at 400 mg /kg; AAEC1, *A. abyssinica* essential oil at 100 mg/kg; AAEC2, *A. abyssinica* essential oil at 200 mg/kg; AAEC4, *A. abyssinica* essential oil at 400 mg/kg.

Comparing the levamisole treated mice to the control vehicle, the HAT level increased by 62.33%. Figure 2 illustrates how *L. sativum* essential oil had a little stronger effect on HAT than *A. abyssinica* at equivalent doses.

As shown in Table 3, there was significantly less footpad edema in the group treated with cyclophosphamide compared to the control group (p<0.001). Only the *L. sativum* 400 mg/kg essential oil test group had a statistically

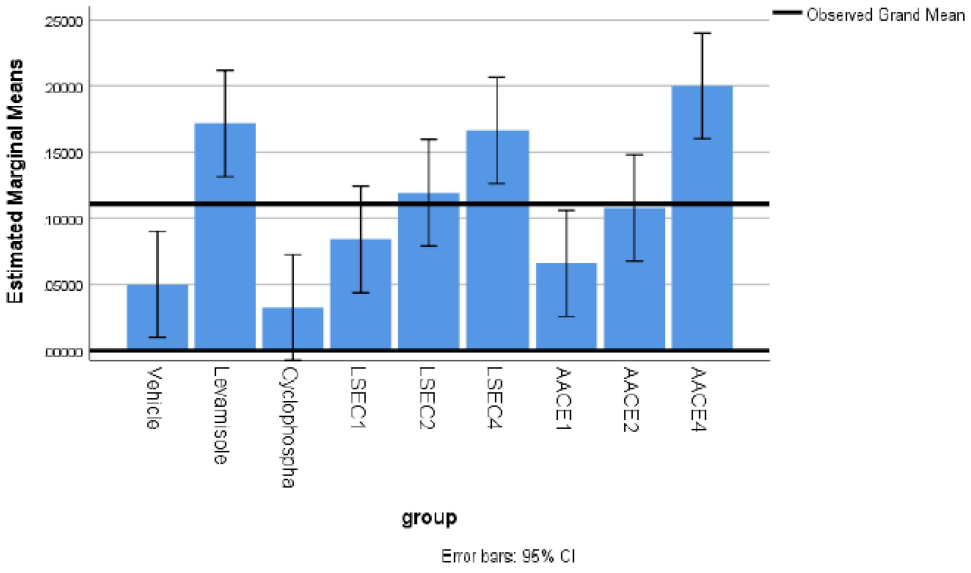

Figure 1 Estimated marginal means of carbon clearance rate of essential oil of *A. abyssinica* and *L. sativum* in mice. The result is expressed as mean ± standard error of the mean (n =5). The error bars represent 95% CI of the mean generated during one-way ANOVA post Hoc Tukey's test (p ≤ 0.05). The grand mean is the average of the means of 9 subsamples with n =5 data points. The true population mean will lay in-between the upper and lower point of the 95% confidence interval in the error bar and the smaller the CI, the higher precision of the sample mean for groups indicated on the horizontal axis. If the error bars overlap quite a bit in the range, there is no statistically significant difference between or among the average though slight difference in the average response rate.

Abbreviations: AACE1, *A. abyssinica* essential oil at 100 mg/kg; AACE2, *A. abyssinica* essential oil at 200 mg/kg; AACE4, *A. abyssinica* essential oil at 400 mg/kg; LSEC1, *L. sativum* essential oil at 100 mg /kg; LSEC2, *L. sativum* essential oil at 200 mg /kg; LSEC4, *L. sativum* essential oil at 400 mg /kg; Cyclophospha, Cyclophosphamide.

Table 2 The Effect of Essential Oils of *A. abyssinica* and *L. sativum* on Humoral Antibody Titer (HAT) in Mice

Groups	Dose (mg/kg)	HAT (Mean ± SEM)	Change (%)	95% Confidence Interval	
				Lower Bound	Upper Bound
Vehicle	———	32.828±1.951		28.871	36.785
Levamisole	50	87.148±1.951^{V***C***LS1***LS2***A1***A2***}	↑ 62.33	83.191	91.105
Cyclophosphamide	30	25.806±1.951	↓ 27.21	21.849	29.763
LSEC1	100	48.114±1.951^{V***L***C***LS4***A4***}	↑31.77	44.157	52.071
LSEC2	200	52.116±1.951^{V***L***C***LS4***A4***}	↑37.00	48.159	56.073
LSEC4	400	84.668±1.951^{V***C***LS1***LS2***A1***A2***}	↑61.23	80.711	88.625
AAEC1	100	46.676±1.951^{V***L***C***LS***A4***}	↑29.66	42.719	50.633
AAEC2	200	49.730±1.951^{V***L***C***LS4***A4***}	↑33.98	45.773	53.687
AAEC4	400	81.816±1.951^{V***C***LS1***LS2***A1***A2***}	↑59.87	77.859	85.773

Notes: Results are expressed as mean ± SEM; n=5. V= Compared to the vehicle, C= Compared to cyclophosphamide, A1= Compared to *A. abyssinica* essential oil at 100 mg/kg, A2= Compared to *A. abyssinica* essential oil at 200 mg/kg, A4= Compared to *A. abyssinica* essential oil at 400 mg/kg, L= Compared to levamisole, LS1= Compared to *L. sativum* essential oil at 100 mg /kg, LS2= Compared to L. sativum essential oil at 200 mg /kg and LS4= Compared to *L. sativum* essential oil at 400 mg /kg. ***P<0.001.
Abbreviations: SEM, Standard error of the mean; LSEC1, *L. sativum* essential oil at 100 mg /kg; LSEC2, *L. sativum* essential oil at 200 mg /kg; LSEC4, *L. sativum* essential oil at 400 mg /kg; AAEC1, *A. abyssinica* essential oil at 100 mg/kg; AAEC2, *A. abyssinica* essential oil at 200 mg/kg; AAEC4, *A. abyssinica* essential oil at 400 mg/kg.

significant ($p<0.01$) increase in mouse footpad swelling as compared to the model group. Lower doses of *L. sativum* essential oil did, however, not statistically significantly, increase the footpad edema. Compared to the model group, the essential oil of *A. abyssinia* at all dose levels caused a non-statistically significant increase in mouse footpad swelling. *A. abyssinica* essential oil had a stronger effect on footpad edema at lower dosages (100 and 200 mg/kg) than *L. sativum* at comparable doses (Figure 3).

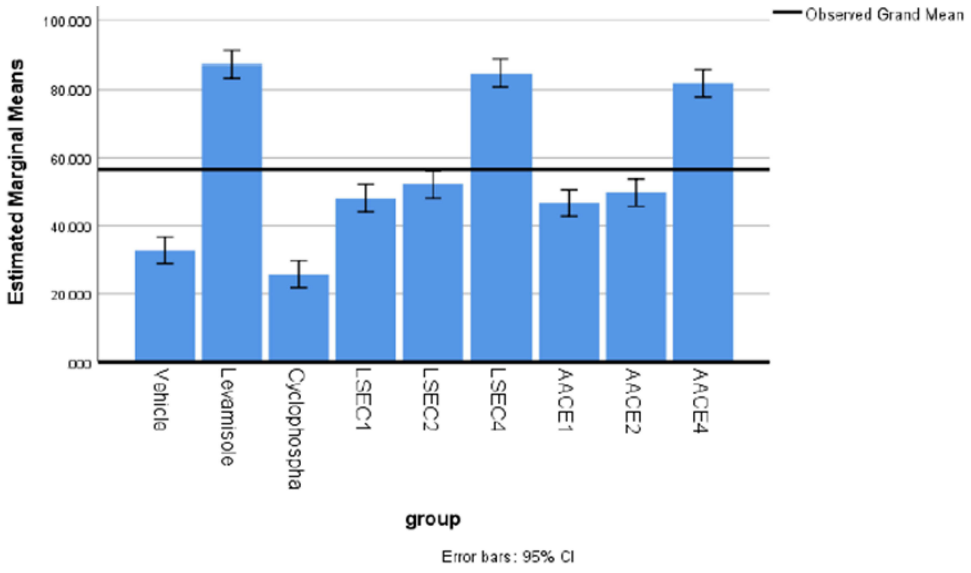

Figure 2 Estimated marginal means of humoral antibody titer of essential oil of *A. abyssinica* and *L. sativum* in mice. The result is expressed as mean ± standard error of the mean (n =5). The error bars represent 95% CI of the mean generated during one-way ANOVA post Hoc Tukey's test ($p \leq 0.05$). The grand mean is the average of the means of 9 subsamples with n =5 data points. If the error bars overlap quite a bit in the range, there is no statistically significant difference between or among the average though slight difference in the average response rate.
Abbreviations: AACE1, *A. abyssinica* essential oil at 100 mg/kg; AACE2, *A. abyssinica* essential oil at 200 mg/kg; AACE4, *A. abyssinica* essential oil at 400 mg/kg; LSEC1, *L. sativum* essential oil at 100 mg /kg; LSEC2, *L. sativum* essential oil at 200 mg /kg; LSEC4, *L. sativum* essential oil at 400 mg /kg; Cyclophospha, Cyclophosphamide.

Table 3 The Effects of Essential Oils of *A. abyssinica* and *L. sativum* on Delayed Type Hypersensitivity in Mice

Groups	Dose (mg/kg)	Increase Thickness Footpad (Mean ± SEM)	95% Confidence Interval	
			Lower Bound	Upper Bound
Vehicle	———	1.498±.070	1.356	1.640
Levamisole	50	1.066±.070	0.924	1.208
Cyclophosphamide	30	0.766±.070^{V}***	0.624	0.908
LSEC1	100	0.894±.070	0.752	1.036
LSEC2	200	0.960±.070	0.818	1.102
LSEC4	400	1.106±.070^{C}**	0.964	1.248
AAEC1	100	0.974±.070	0.832	1.116
AAEC2	200	0.986±.070	0.844	1.128
AAEC4	400	1.042±.070	0.900	1.184

Notes: Results are expressed as mean ± SEM; n=5. V= Compared to the vehicle, C= Compared to cyclophosphamide.**P<0.01, ***P<0.001.

Abbreviations: SEM, Standard error of the mean; LSEC1, *L. sativum* essential oil at 100 mg /kg; LSEC2, *L. sativum* essential oil at 200 mg /kg; LSEC4, *L. sativum* essential oil at 400 mg /kg; AAEC1, *A. abyssinica* essential oil at 100 mg/kg; AAEC2, *A. abyssinica* essential oil at 200 mg/kg; AAEC4, *A. abyssinica* essential oil at 400 mg/kg.

A. abyssinica essential oil at 200 and 400 mg/kg significantly increased the level of thymus index compared to the model group (p<0.05 and 0.01 respectively). The levamisole group has experienced the highest increase in TI (p<0.001) (Figure 4). Essential oil of *L. sativum* at 400 mg/kg also increased the level of TI at a significant level when compared to the model group (Table 4).

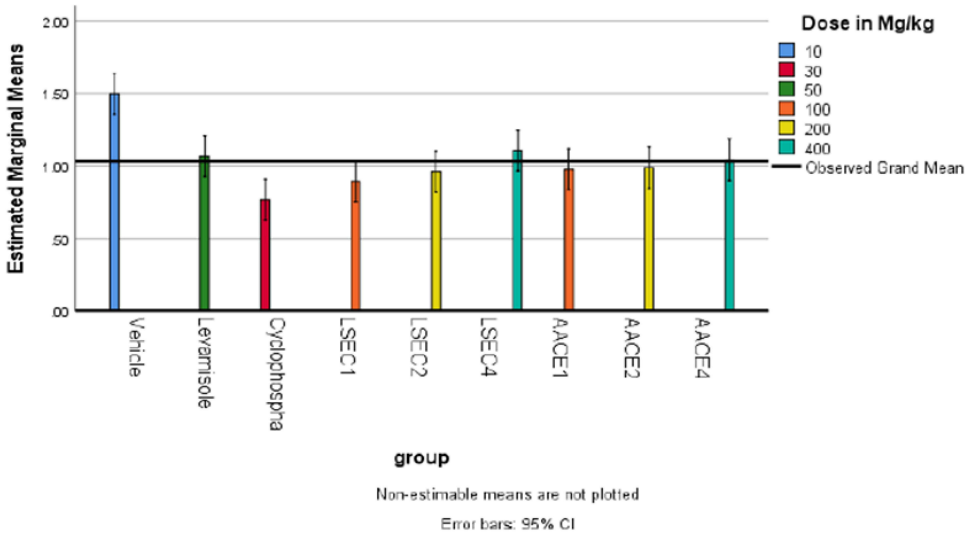

Figure 3 Estimated marginal means of delayed type hypersensitivity response of essential oil of *A. abyssinica* and *L. sativum* in mice. The result is expressed as the mean ± standard error of the mean (n =5). The error bars represent 95% CI of the mean generated during one-way ANOVA post Hoc Tukey's test (p ≤ 0.05). The grand mean is the average mean of 9 subsamples with n =5 data points. It represents the overall mean of a set of 9 subsamples. The true population mean will lay in-between the upper and lower point of the 95% confidence interval in the error bar and the smaller the CI, the higher precision of the sample mean for groups indicated on the horizontal axis. If the error bars overlap quite a bit in the range, there is no statistically significant difference between or among the average though slight difference in the average response rate.
Abbreviations: AACE1, *A. abyssinica* essential oil at 100 mg/kg; AACE2, *A. abyssinica* essential oil at 200 mg/kg; AACE4, *A. abyssinica* essential oil at 400 mg/kg; LSEC1, *L. sativum* essential oil at 100 mg /kg; LSEC2, *L. sativum* essential oil at 200 mg /kg; LSEC4, *L. sativum* essential oil at 400 mg /kg; Cyclophospha, Cyclophosphamide.

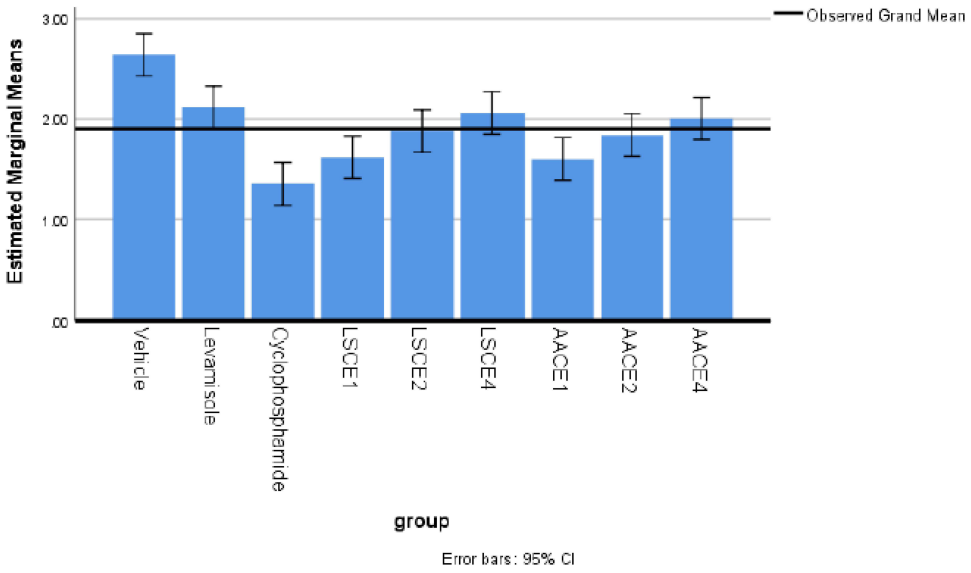

Figure 4 Estimated marginal means of thymus index of essential oil of *A. abyssinica* and *L. sativum* in mice. The result is expressed as mean ± standard error of the mean (n =5). The error bars represent 95% CI of the mean generated during one-way ANOVA post Hoc Tukey's test ($p \leq 0.05$). The grand mean is the average mean of 9 subsamples with n =5 data points. The true population mean will lay in-between the upper and lower point of the 95% confidence interval in the error bar and the smaller the CI, the higher precision of the sample mean for groups indicated on the horizontal axis. If the error bars overlap quite a bit in the range, there is no statistically significant difference between or among the average though slight difference in the average response rate.

Abbreviations: AACE1, *A. abyssinica* essential oil at 100 mg/kg; AACE2, *A. abyssinica* essential oil at 200 mg/kg; AACE4, *A. abyssinica* essential oil at 400 mg/kg; LSEC1, *L. sativum* essential oil at 100 mg /kg; LSEC2, *L. sativum* essential oil at 200 mg /kg; LSEC4, *L. sativum* essential oil at 400 mg /kg; Cyclophospha, Cyclophosphamide.

The spleen index was also significantly lower ($p<0.001$) in the model group compared to the vehicle (Table 5). The spleen index in mice was improved by both essential oils only at the highest dose levels (400 mg/kg) in comparison to the model group. This result was comparable to mice treated with levamisole (Figure 5).

Table 4 The Effect of Essential Oils of *A. abyssinica and L. sativum* on Thymus Index (TI) in Mice

Groups	Dose (mg/kg)	TI (Mean ± SER)	95% Confidence Interval	
			Lower Bound	Upper Bound
Vehicle	———	2.638± 0.105	2.426	2.850
Levamisole	50	$2.112\pm.105^{C***}$	1.900	2.324
Cyclophosphamide	30	$1.350\pm 0.105^{V***}$	1.138	1.562
LSEC1	100	1.616± 0.105	1.404	1.828
LSEC2	200	$1.876\pm.105^{C*}$	1.664	2.088
LSEC4	400	$2.060\pm.105^{C+}$	1.848	2.272
AAEC1	100	1.600±.10	1.388	1.812
AAEC2	200	1.838±.105	1.626	2.050
AAEC4	400	$2.004\pm.105^{C**}$	1.792	2.216

Notes: Results are expressed as mean ± SEM; n=5. V= Compared to the vehicle, C= Compared to cyclophosphamide. *P<0.05, **P<0.01, ***P<0.001.

Abbreviations: SEM, Standard error of the mean; LSEC1, *L. sativum* essential oil at 100 mg /kg; LSEC2, *L. sativum* essential oil at 200 mg /kg; LSEC4, *L. sativum* essential oil at 400 mg /kg; AAEC1, *A. abyssinica* essential oil at 100 mg/kg; AAEC2, *A. abyssinica* essential oil at 200 mg/kg; AAEC4, *A. abyssinica* essential oil at 400 mg/kg.

Table 5 The Effect of Essential Oils of *A. abyssinica and L. sativum* on Spleen Index (SI) in Mice

Groups	Dose (mg/kg)	SI (Mean ± SER)	95% Confidence Interval	
			Lower Bound	Upper Bound
Vehicle	———	3.586± 0.156	3.271	3.901
Levamisole	50	2.866±.156	2.551	3.181
Cyclophosphamide	30	2.342± 0.156^{V***}	2.027	2.657
LSEC1	100	2.340± 0.156	2.023	2.645
LSEC2	200	2.404±.156	2.089	2.719
LSEC4	400	2.784±.156	2.469	3.099
AAEC1	100	2.276±.156	1.961	2.591
AAEC2	200	2.406±.156	2.091	2.721
AAEC4	400	2.902±.156	2.587	3.217

Notes: Results are expressed as mean ± SEM; n=5. V= Compared to the vehicle. ***P<0.001.
Abbreviations: SEM, Standard error of the mean; LSEC1, *L. sativum* essential oil at 100 mg /kg; LSEC2, *L. sativum* essential oil at 200 mg /kg; LSEC4, *L. sativum* essential oil at 400 mg /kg; AAEC1, *A. abyssinica* essential oil at 100 mg/kg; AAEC2, *A. abyssinica* essential oil at 200 mg/kg; AAEC4, *A. abyssinica* essential oil at 400 mg/kg.

Discussion

The mononuclear phagocytotic activity is a sign for non-specific immunity. The phagocytosis of carbon particles is caused by stimulation of the reticuloendothelial system.[37] The carbon clearance method can be used to calculate the macrophage phagocytic index, which indicates the phagocytotic function.[36]

In our study, mice treated with *A. abyssinica* and *L. sativum* essential oils had a higher rate of carbon elimination compared to the vehicle or cyclophosphamide. These essential oils' enhanced macrophage phagocytic index may be due to their antioxidant properties.[11,21] The immune system is strengthened by antioxidants.[23] The stimulation of the hematological system and lymphocyte differentiation may be associated with the increased carbon clearance rate.[38] Both essential oils showed a dose-dependent effect on mice's ability to remove carbon particles. The 400 mg/kg had the greatest impact on the

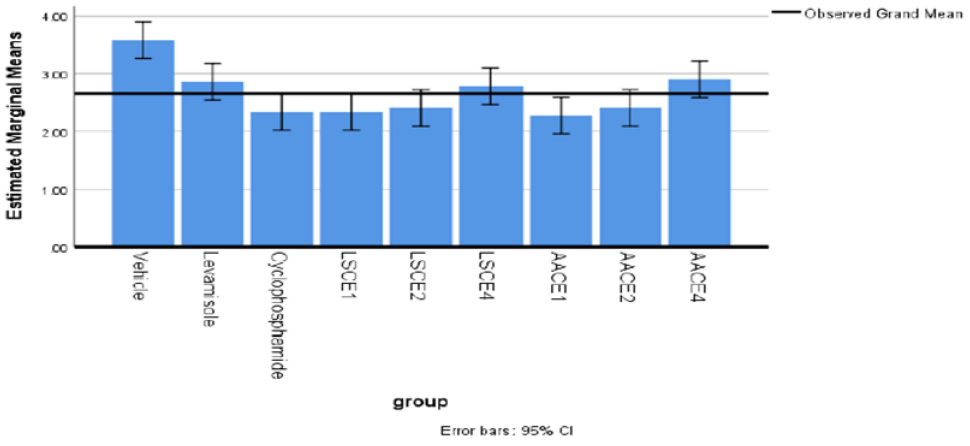

Figure 5 Estimated marginal means of spleen index of essential oil of *A. abyssinica* and *L. sativum* in mice. The result is expressed as mean ± standard error of the mean (n =5). The error bars represent 95% CI of the mean generated during one-way ANOVA post Hoc Tukey's test (p ≤ 0.05). The grand mean is the average of the means of 9 subsamples with n =5 data points. The true population mean will lay in-between the upper and lower point of the 95% confidence interval in the error bar and the smaller the CI, the higher precision of the sample mean for groups indicated on the horizontal axis. If the error bars overlap quite a bit in the range, there is no statistically significant difference between or among the average though slight difference in the average response rate.
Abbreviations: AACE1, *A. abyssinica* essential oil at 100 mg/kg; AACE2, *A. abyssinica* essential oil at 200 mg/kg; AACE4, *A. abyssinica* essential oil at 400 mg/kg; LSEC1, *L. sativum* essential oil at 100 mg /kg; LSEC2, *L. sativum* essential oil at 200 mg /kg; LSEC4, *L. sativum* essential oil at 400 mg /kg; Cyclophospha, Cyclophosphamide.

outcome. This might be as a result of the availability of several secondary metabolites in larger concentrations, which may enhance phagocytic action in an additive or synergistic manner.[39] When compared to L. sativum and levamisole treated groups, the essential oil of *A. abyssinica* at 400 mg/kg resulted in the highest carbon clearance rate. This may imply that the essential oil of *A. abyssinica* contains a higher concentration of immunostimulant phytochemicals. In a prior study, mice given extracts of *Bituminaria bituminosa* or *Ambrosia maritima* showed an increase in carbon clearance. The immunostimulatory effects of these extracts were dosage dependent. The larger effects were evident at the highest doses.[40]

One important metric used to examine the humoral response is the titer of the hemagglutination antibody. The humoral immune response is mediated by antibody molecules produced by plasma cells.[41] In the current investigation, compared to the vehicle and cyclophosphamide treated groups, essential oils of *A. abyssinica* and *L. sativum* raised the amount of HAT to SRBC antigen at all dose levels. This demonstrates that both essential oils have immunostimulant effects. Increased reactivity of macrophages, a subpopulation of T and B lymphocytes involved in the manufacture of antibodies, is linked to humoral response stimulation.[42] The increased antibody titer suggests that the essential oils of *A. abyssinica* and *L. sativum* may have an impact on the Th-2 pathway. Promoting the production and release of IL-4 while inhibiting the production of IL-12 could result in this outcome.[41] The inclusion of flavonoids, phenolics, alkaloids, saponins, glycosides, coumarins, fixed oils, proteins, vitamins, and minerals in these essential oils[12,22] that stimulate the development of macrophages, B lymphocytes, and T lymphocytes[1] may be the cause for elevation of HAT. The maximal (84.668±1.951) statistically significant ($p<0.001$) mean secondary HAT to SRBC was generated by *L. sativum* essential oil at 400 mg/kg, increasing the level of HAT by 61.23%. At 400 mg/kg, the *A. abyssinica* essential oil raised the level of secondary HAT to SRBC by 59.87% when compared to the vehicle control group. The mean secondary HAT to SRBC antigen was increased by both essential oils at the highest doses. This increase was comparable to the common medication levamisole (62.33%). Comparing *L. sativum* essential oil to *A. abyssinica* at similar doses, the effect on HAT was substantially larger for *L. sativum*. This may be caused by *L. sativum* having a larger concentration of compounds that strongly encourage the growth of blood cells engaged in humoral immunity. According to one study, *Nigella sativa* has a somewhat stronger agglutinating effect than *L. sativum* extract.[22] Another study found that rats' antibody titer increased in a dose-related manner after receiving 50, 100, 200, 400, and 800 mg/kg of the essential oils of geranial, geranial acetate, gingerol, and eugenol for 15 days.[43] In mice, *Mangifera indica* and *Curcuma domestica* were shown to have immunostimulant properties in a Malaysian study. The animal groups that were administered these treatments exhibited a notable rise in the haemagglutination titer, indicating a broad stimulation of the humoral immune response, compared to the leaves of *Mangifera indica*, the rhizome of *Curcuma domestica* exhibited more humoral immune stimulation.[44]

Hypersensitivity of the delayed kind is protective. It is recommended to evaluate the impact of essential oils on cellular immunity using SRBC induced delayed type hypersensitivity.[45] The footpad swelling method[36] may be used to gauge the impact on delayed type hypersensitivity. Among the test groups, only the *L. sativum* essential oil test group at 400 mg/kg demonstrated a statistically significant ($p<0.01$) rise in mouse footpad swelling as compared to the control group. This could be as a result of the greater concentration of secondary metabolites found in *L. sativum* essential oil that significantly induces a localized aberrant reactive inflammation characterized by cell degeneration and necrosis.[45] However, at lower dosages (100 and 200 mg/kg), A. *abyssinica* essential oil increased footpad swelling more than *L. sativum* at comparable doses, but the difference was not statistically significant. This demonstrates the *A*. abyssinica essential oil's increased potency on delayed type hypersensitivity in mice.

The most commonly used indexes for assessing an organism's overall immunological function are the thymus and spleen indices.[46] In this study, both essential oils raised the TI of mice as compared to the model group at all test levels. In comparison to the levamisole group, this effect was reduced. Only the highest doses (400 mg/kg) of both essential oils increased the spleen index in mice compared to the model group. This finding was comparable to levamisole group.

Our results are consistent with prior researches showing that plants high in flavonoids and polyphenols have significant immune-stimulating effects.[47–51]

The current research supports that the essential oils of *A. abyssinica* and *L. sativum* may have in vivo immunostimulant effects in mice.

Conclusion

It can be inferred that the essential oils of *L. sativum* and *A. abyssinica* have immunostimulant properties. In mice, these oils can increase carbon clearance rate, humoral antibody titer, delayed type hypersensitivity, thymus and spleen indices. It is possible to plan future research on molecular mechanisms of action, in-vitro immunomodulatory assays, comprehensive toxicity profiles, and phytochemical analysis.

Abbreviations

EMM, Estimated Marginal Means; DTH, Delayed Type Hypersensitivity; HAT, Humoral Antibody Titer; LD50, Median Lethal Dose; OD, Optical Density; OGM, Observed Grand Mean; RPM, Revolution per Minute; SI, Spleen Index; TI, Thymus Index.

Data Sharing Statement

All the data are found in this paper. The corresponding author of this manuscript will provide more information upon reasonable request. Email: kassh2009@gmail.com.

Ethical Approval and Consent to Participate

The study was approved by Institutional Review Board of Debre Berhan University in Ethiopia. A letter of clearance was obtained. The plant materials were collected considering relevant national and international guidelines and legislations.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

No competing interests exist.

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