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In Vivo Immune Study of *Achillea Fragrantissima* Extract *Versus* Echinaid and Endoxan in Wistar Rats

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Abstract

Immunity is a major concept in human nutrition. Immuno compromised individuals are at risk for serious infection as COVID-19 which is directed the researchers to use the immuno modulatory plants for prophylaxis. This study was designed to assess the immune response of Wistar rats administrated *Achillea fragrantissima* (*A. fragrantissima*) extract versus Endoxan (immuno suppressive) and Echinaid (immune stimulating). Fifty rats were assigned into 5 groups:(1)control, (2) injected intraperitoneal (i/p) with Endoxan 90 mg/kg for three successive days, (3) injected with Endoxan as (2) and administrated with Echinaid 300 mg/kg/day. (4,5) injected with Endoxanas (2) and administrated with 300 and 500 mg/kg/ day *A. fragrantissima* extract respectively. On day 17 all groups were challenged with two doses of sheep erythrocytes (SRBC) i/p, 2 weeks intervals. A high dose of *A. fragrantissima* extract achievedan increase in total antioxidant capacity significantly (P<0.05), superoxide dismutase, and a decrease in malondialdehyde. Catalase exerted a significant increase with a low dose of *A. fragrantissima* whereas a high dose had a mild effect. Echinaid and *A. fragrantissima* raised IgM for the first dose of SRBC and Igs and IgG for the second dose significantly (P<0.05). *A. fragrantissima* administration ameliorates cytokines (TNF-α, IL-4) and modulated IL-10 significantly (P<0.05). A high dose of *A. fragrantissima* extract exerted a significant reduction in splenic

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non-cellular viability% and the highest score of the microscopic immune reaction (immunostimulation++++). Splenic histopathology confirmed the present results. The current study highlights that a high dose of *A. fragrantissima* extract is preferred over a low dose to restore immune responses *in vivo.*

Introduction

In the concept of nutrition, the connection between diet and health is a major area of research; however, enhancing immunity is a major interest in the human diet. Indeed, an array of plants and their components hold immuno modulatory properties.[1] Plants containing functional ingredients can be utilized as prophylaxis for preventing infections.[2]

An emerging epidemic of unexplainable pneumonia was recorded,[3] named COVID-19 by World Health Organization. Unfortunately, specific antiviral drugs currently have not been available for treatment,[4] and the vaccines are not accurate 100% besides it is expensive for many countries.[5] To overcome these obstacles, the authorsat Shanghai Changhai Hospital announced that it should speed up research performance on traditional Chinese medicine (TCM) which is used as an alternative remedy.[6] Immuno compromised patients are at high risk for COVID-19 infection which underlies the use of the immuno modulatory plant for prophylaxis and prevention of the disease.[7] Recently, they found that TCM has antivirus, anti-inflammation, through balancing the immune system regulation.[8,9]

Achillea fragrantissima (Forssk.) Sch. Bip. (*A. fragrantissima*) is related to the *Asteraceae* family in Arabic called ''Qaysoom''. Traditionally, it is used as a medical plant in Arabian countries for the treatment of dysfunction of the liver and kidneys, gastrointestinal tract, as well as wound healing due to its antiseptic properties.[10] *Achillea fragrantissima* is used in the treatment of common health problems such as respiratory disease, eye infections, smallpox, fever, diabetes, dysmenorrhea, headache, or fatigue.[11] *Achillea fragrantissima* oil used in many countries as a stomachic, diuretic, anthelmintic, and antispasmodic.[12] It has antimicrobial, anti-inflammatory, anticancer, antifungal, and antibacterial effects. Nevertheless, till now no clinical uses for *A. fragrantissima* are described.[13]

Endoxan (cyclophosphamide) belongs to a group of medicines called antineoplastic or cytotoxic medicines used routinely for chemotherapy and in hematopoietic cell transplantation usually exerts side effects,[14] especially immuno suppressive impacts,[15] and oxidative stress.[16] It causes a sudden and adverse change of proinflammatory and anti-inflammatory cytokines (Th1/Th2) balance,[17] and immunoglobulins.[18]

On the other hand, Echinaid (*Echinacea purpurea*) supplement boosts the immune system. Echinaid has been used to prevent and treat upper respiratory infections.[19] Recently, many researchers settled that *Echinacea purpurea*could modulate immune functions in both animals and humans, because of its glycoproteins, alkyl amides, and polysaccharides compounds which have antioxidative, anti-inflammatory, and immuno modulatory effects.[20]

Anyway, most of the results published with *A. fragrantissima* extract have been conducted *in vitro*, little information exists *in vivo* and with oral administration. According to our knowledge, no study recorded the immune responses of the experimental animal that received *A. fragrantissima* taking into its consideration a comparison with immuno suppressiveand immune-stimulating standards. Therefore, in the present study, it is wise to take into consideration, the detection of bioactive phytochemical constituents and antioxidant activities of *A. fragrantissima* extract. Furthermore, the immune modulation of *A. fragrantissima* extract will standardize with a positive immune stimulant (Echinaid) and negative immune suppressive (Endoxan). In this context, the current work is concerned with serum immunoglobulins: total immunoglobulins (Igs), immunoglobulins M (IgM), and immunoglobulins G(IgG) as well as cytokines (TNF-α, IL4, and IL10) changes with oral administration of *A. fragrantissima* extract. Histopathological investigation of the spleen,

as one of the main organs involved in immune response, will be performed.

Materials and Methods

Ethical Standard

Qassim University Committee, Kingdom of Saudi Arabia, approved the current work as planned by the International Animal Ethics Committee under the number "cavm-2018-1-14-S-3478".

Preparation of *Achillea Fragrantissima* Extract for Oral Administration

The Aerial parts of *A. fragrantissima* with a voucher specimen (AF-2008-51) were dried for 10 days at room temperature. The dried plant was ground into powder, then extracted with 80% ethanol (Arkan, Germany): and 20% distilled water using the Soxhlet apparatus (Shanghai Heqi Glassware Co., Ltd. China) for 18 hours.[21] The ethanol extract was evaporated and concentrated in a rotary evaporator (KNF Technology Shanghai Co., Ltd. China). The crude extract was diluted with distilled water to a concentration of 100 mg/ml.

Preparation of Sheep Erythrocytes (SRBCs)

Erythrocytes were extracted from healthy sheep blood as previously collected in sterile Alsevar's solution (20.0 gm D-glucose, 8.0 gm sodium chloride, 4.2 gm sodium citrate, and distilled water up to 1000.0 ml.). The mixture was centrifuged 3 times at low speed for isolation of erythrocytes.[22] The obtained cells were adapted to be in a concentration equivalent to 100 µl phosphate buffer saline (PPS) pH 7.2 containing 1×10^5/mm3 cells. Cells were adjusted by using a hemocytometer.

Animals

Adult healthy Wistar rats about 6 weeks of age weighed (170–200 g) were get from the laboratory center of the University of King Saud, Riyadh, KSA. Rats were put in a fitted rearing room, College of Agriculture and Veterinary Medicine, Qassim University, KSA. Animal acclimatization was performed by keeping rats in suitable cages at stable room temperature (22±2 ^{o}C) under a light/dark cycle photoperiod[12] hrs. with free available fresh water and a commercial diet purchased from General Company of Feed Mills. The rat's diet was formulated to be furnished with the nutrients recommended by National Research Council (NRC).[23] We did our best to minimize animal's suffering and reduce pain. After one week of acclimatization, the experiment was started.

Experimental Design and Sampling

Fifty animals were assigned into five groups ten per each as follows; Group (1): untreated control group. Rats in all other groups (2-5) were injected intraperitoneal (i/p) with Endoxan containing 200 mg cyclophos phamide (Baxter Oncology GmbH-kantstrassa 2- D-33790 Halle, Germany) as a standard immuno-suppressive, in a dose of 90 mg/kg for three successive days.[18] Group (2): Endoxan only. Group (3): Endoxan plus Echinaid (ESI srl Via delle industrie 1 Albissola Marina (SV) ITALY esi.it) containing *Echinacea purpurea* dry powder 375 mg/capsule as a standard immuno-stimulant, in a dose of 300 mg/kg/day orally.[24] Groups (4 and 5): Endoxan and administrated with 300 and 500 mg/kg body weight per day *A. fragrantissima* extract orally suspended in saline respectively. *A. fragrantissima* extract doses were chosen to be within the safe and common range without any toxicity.[21,25] On day 17 of the experiment rats in all groups (1-5) were challenged with the first dose of SRBCs (0.5 ml SRBCs suspensions containing 1×10^8/mm^3 cells i/p) followed by a second booster dose after 2 weeks i.e., at day 31 of the experiment (Table 1).

The experiment was conducted for 38 days. Rats were anesthetized with ether to collect blood samples pre-and post- the second dose of SRBC challenge atone-weekintervalsin all experimental groups, i.e., on days 24 and 38 of an experiment to follow up antibodies' titers. Whilst the blood collected post-second dose of SRBC challenge was used to determine the biochemical analysis suggested in the present study. The spleen was gently removed from 3 animals in each group, for the determination of splenic non-viable cell numbers in percentage and counting the score of microscopic immune reaction of the spleen as well as for histopathological examination as mentioned below.

Table 1: Grouping and designing of the current experiment.

Groups	Day 1-3 (90 mg/kg Endoxan)	Day 1-17	Day 17	Day 24	Day 31	Day 38
Group (1)	-	-	Sheep erythrocytes (SRBCS) challenge	Blood samples	Sheep erythrocytes (SRBCS)challenge	Blood and spleen samples
Group (2)	√	-				
Group (3)	√	300 mg/kg Echinaid/day				
Group (4)	√	300 mg/kg *Achillea fragrantissima*/day				
Group (5)	√	500 mg/kg*Achillea fragrantissima* /day				

Measurements

Gas Chromatography-Mass Spectral Analysis (GC/MS)

The aerial parts of *A. fragrantissima* were subjected to extraction using the same steps previously mentioned in preparation of *A. fragrantissima* extract for oral administration but using methanol 99.9% instead of 80% ethanol. The methanolic extract of the plant was analyzed qualitative and quantitative separately to identify the bioactive constituents of *A. fragrantissima.*[26] The process was done using Agilent Gas Chromatography (Model 6890N coupled to 5973 Mass Selective Detector (MSD), (USA). The relative percentage amount of each component was calculated by comparing its average peak area to the total areas using software adopted to handle mass spectra and chromatograms (Turbo Mass Version 5.2).

Antioxidant Activities of *Achillea fragrantissima*

The plant was extracted with 50% aqueous ethanol by stirring for 3 minutesat 25,000 rpm using a homogenizer (IKA, Germany). Samples were then centrifuged at 3500 rpm for 10 min to get the supernatants which were used for antioxidant analyses.[27] Antioxidant activities of plant extract were detected for total phenolic content (TPC), 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), and 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS). The total phenolic content was estimated by the Folin-Ciocalteu method using gallic acid as the standard.[28] The 2, 2-Diphenyl-1-picrylhydrazyl was determined using a modified method of Brand-Williams to determine antioxidant activity.[29] The 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid was estimated using a modified method of Reto determine antioxidant activity using Trolox as the standard.[30]

Serum Antioxidant Activities and Lipid Peroxidation Biomarker

The antioxidant activities of serum were assayed by detecting the total antioxidant capacity (TAC), superoxide dismutase enzyme (SOD), and catalase enzyme (CAT) contents. Meanwhile, the lipid peroxidation biomarker is represented by malondialdehyde (MDA). The TAC,SOD, CAT, and MDA were assessed using Biodiagnostic kits (Cairo, Egypt) Cat. No. (TA 26 14, SD 26 22, CA 26 18, and MD 26 30 respectively). The absorbencies of TAC, SOD, CAT, and MDA were spectrophotometrically measured at 340 nm, 560 nm, 520 nm, and 534 nm respectively.

Table 2: Gas chromatography-mass spectral (GC/MS) components of *Achilleafragrantissima*

Retention time (minutes)	Peak % area	Name	Molecular formula
3.424	11.18	Thujone	C10H16O
4.910	2.42	4-Cyclohepten-1-amine	C7H13N
5.358	12.97	1,8-Cineole	C10H18O
6.647	8.55	Artemisia ketone	C10H16O
7.028	22.65	Camphor	C10H16O
8.156	2.05	1H-Cyclopenta[1,3]cyclopropa[1,2]benzene,	C15H24
8.603	7.45	Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methylene	C15H24
10.068	1.20	Methyl jasmonate	C13H20O3
10.211	1.28	2-Naphthalenemethanol, decahydro-α,α,4a-trimethyl-8-methylene-,	C15H26O
13.346	4.01	5-Isopropyl-2,8-dimethyl-9-oxatricyclo[4.4.0.0(2,8)]decan-7-one	C14H22O2
13.512	3.38	9-t-Butyltricyclo[4.2.1.1(2,5)]decane-9,10-diol	C14H24O2
15.326	1.07	3α,4β-Dihydroxy-1,5,7α(H),6β(H)-guai-10(15),	C15H20O4
16.299	0.96	Dihydroxanthin	C17H24O5
16.928	1.59	6β,19-Cycloandrost-4-ene-3,17-dione	C19H24O2
19.211	0.84	Folic Acid	C19H19N7O6
19.721	5.35	Androstan-17-one, 3-ethyl-3-hydroxy-, (5α)-	C21H34O2
23.943	2.06	Tris(2,6-dimethylphenyl)borane	C24H27B

Table 3: Antioxidant activity of the *Achillea fragrantissima*

Parameters	TPC	DPPH	ABTS
A. fragrantissima	1446.59±40.51	77.58±7.10	67.57±4.17

Total Phenolic Content (TPC) expressed as mg gallic acid equivalents (GAE) per 100 g, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) express percentage inhibition ofthe DPPH radical, and 2, 2'-Azino- Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) expressmg Trolox equivalents. Mean± Standard error (SE).

Serum Total Immunoglobulins (Igs), Immunoglobulins M (IgM), and Immunoglobulins G (IgG) Titers (Log2)

Detecting the antibodies against SRBC was performed using the ELISA method to measure the humoral immune response. ELISA was used to measure total Igs, IgM, and IgG anti-SRBC membrane antibody titers in sera.[31] Hemoglobin-free SRBC membranes were prepared, and the protein content of the solution was measured using SPECTRUM kits Cat. No. 08-700-131. ELISA plates were coated with prepared SRBC membrane.[32] Each serum sample was then added and incubated at 4°C for 12 hs. The use of mercaptoethanol-resistant IgG and sensitive IgM was followed as previously described.[33]

Cytokines Assay (TNF-α, IL-4, and IL-10)

The pro-inflammatory cytokine determination, represented by TNF-αwas performed using ELISA kits (Assaypro, 30 Triad South Drive, St Charles MO 63305, USA).The anti-inflammatory cytokines including Interleukin-4 (IL4) and Interleukin-10 (IL10) were assayed by ELI-SA kit (Cusabio Biotech Co., Ltd. Lot: 004152652, Wuhan, China). The manufacturer's instructions were followed, and the color change was measured spectrophotometrically at a wave length of 450 nm.

Spleen Non-Cellular Viability %

Spleen specimens collected at the end of the experiment were divided into two parts. The first part was exposed to erythrocytes lysis with warm isotonic ammonium chloride lysing solution to use for determination of non-viable cell number % using trypan blue dye exclusion method.[34] Following the next equation:

Spleen non-cellular viability % = Number of dead cells/ Total cell number X 100

Histopathology of the spleen

The second part of spleen specimens was preserved using a 10% neutral buffered formalin aqueous solution and processed using routine paraffin wax, then stained with H & E (Hematoxylin, and Eosin)[35] for histological observations. The score of the microscopic immune reaction of the spleen which is expressed in (– and +) marks has been counted.

Statistical Analysis

Values of data were illustrated as means ± standard errors. A simple one-way analysis of variances (ANOVA) test was used for each measured parameter. Post hoc analysis using the Mann-Whitney test was performed to compare the control with the Endoxan groupand between the Endoxan and other experimental groups with P<0.05 reflecting a statistical difference.

Results

Chromatographic Analysis (GC–MS) of *Achillea fragrantissima*

The major phytochemical constituents of *A. fragrantissima* extract were determined by GC–MS found in (Table 2). Camphor, 1,8-Cineole, Artemisia ketone, Thujone, and Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methylene were the main bioactive constituents of *A. fragrantissima*.

Antioxidant Activities of *Achillea fragrantissima*

The antioxidant activities of *A. fragrantissima* were 1446.59±40.51, 77.58±7.10, and 67.57±4.17 for TPC, DPPH, and ABTS respectively(Table 3).

Serum Antioxidants Enzyme Activities and Malondialdehyde

Rats treated with Endoxan without plant extract and challenged with SRBC (group 2) exert a significant (P<0.05) reduction in the TAC, SOD, and CAT comparable to the control group. On the contrary, Echinaid which was supplemented as an immune stimulant standard recorded significant (P<0.05) alleviation of the adverse effect of Endoxan. Echinaid and *A. fragrantissima* extracts in both doses (300 and 500) mg/kg showed significant (P<0.05) elevation in SOD. A high dose of *A. fragrantissima* extract (500 mg/kg) ameliorated TAC significantly (P<0.05) in comparison to the Endoxan group,

Table 4: Effect of *Achillea fragrantissima* extract on serum antioxidants enzyme activities and malondialdehyde.

Groups/ Parameters	TAC (mM/l)	SOD (U/ml)	CAT (u/l)	MDA (nmol/ml)
(1) Control	3.11±0.22	386.2±8.11	365.1±4.64	0.528±0.041
(2) Endoxan	2.14±0.39*	253.7±7.04*	265.4±5.75*	0.981±0.034*
(3) Echinaid	3.71 ±0.42[a]	333.6±5.10[a]	361.5±6.88[a]	0.362±0.101[a]
(4) *A. fragrantissima* (300 mg/kg)	2.54 ±0.15	369.0±5.19[a]	371.8±5.51[a]	0.870±0.06
(5) *A. fragrantissima* (500 mg/kg)	3.82 ±0.42[a]	308.3±5.28[a]	274.4±8.45	0.463±0.032[a]

Means having mark* in the same column have significant values at (P<0.05) compared to the control SRBC group. Means having letter ain the same column has significant values at (P<0.05) compared to the Endoxan group challenged with SRBC. Total antioxidant capacity (TAC); Superoxide dismutase enzyme (SOD); Catalase enzyme (CAT); Malondialdehyde (MDA). mean ± Standard error (SE)

where it showed non-significant improvement in CAT enzyme (Table 4). Nevertheless, a low dose of *A. fragrantissima* (300 mg/kg) extract supplementation success to increase CAT significantly (P<0.05) whereas, failed to improve TAC significantly.

Serum MDA, which is the final product of lipid peroxidation exerted significant (P<0.05) elevation in group 2, whereas exhibited significant (P<0.05) improvement by Echinaid and 500 mg/kg *A. fragrantissima* extract supplementations. Serum TAC and MDA did not affect by a low dose of *A. fragrantissima* ((300 mg/kg).

Serum Total Immunoglobulins (Igs), Immunoglobulins M (IgM), and Immunoglobulins G (IgG) Titers Titer

There was a drop in all antibodies titer measured which were significant in Igs and IgM pre- 2ry dose of SRBC challenge (at day 24) and in Igs post- 2ry dose of SRBC challenge (at day 38) for the group treated with Endoxan without plant extract comparable to SRBC control. Echinaid and *A. fragrantissima* succeeded in rising IgM (on day 24) of the experiment and Igs and IgG (on day 38) of the experiment significantly (P<0.05) (Table 5). Total Igs significantly (P<0.05) increased because of pre- 2ry dose of SRBC challenge (day 24) in groups received Echinaid, and 500 mg/kg *A. fragrantissim* although this increase was non-significant at a low dose (300 mg/kg). IgG and IgM did not exert any significant increase for pre and post 2ry doses of SRBC challenge for all experimental treatments.

Serum Cytokines (TNF-α, IL-4, and IL-10)

There was a significant elevation in TNF-α level at (P<0.01) in the group treated with Endoxan without plant extract comparable to SRBC control. However, TNF-α levels showed a non-significant reduction with Echinaid and *A. fragrantissima* at two doses (300 and 500mg/kg in comparison to group (2) which received Endoxan only. The level of cytokine IL-4 was significantly (P<0.01) declined in animals receiving Endoxan compared to the control SRBC challenged group. On the other hand, the level of IL-4 was significant (P<0.05) elevated in rats offered to Echinaid and 300 mg/kg *A. fragrantissima* compared to those subjected to Endoxan and challenged with SRBC group. In addition, IL-10 level showed a significant decline (P<0.01) for rats treated with Endoxan and challenged with SRBC comparable with the control SRBC challenged group (P<0.05). However, the level of IL-10 exerted significant elevation for rats treated with *A. fragrantissima* at two doses (300 and 500) mg/kg (Table 6 & Fig. 1).

Table 5: Effect of *Achillea fragrantissima* extract on total anti-SRBC antibodies (Igs), IgM, and IgG titers (log2)

Groups/ Parameters	Sampling days					
	day 24			day 38		
	Total Igs	IgM	IgG	Total Igs	IgM	IgG
(1) Control	6.86 ± 1.33	4.87 ±1.15	2.15 ±0.66	7.76 ±0.64	2.75 ±0.54	4.91 ±0.71
(2) Endoxan	3.98 ±1.11*	2.87 ±0.35*	1.88 ±0.46	4.20 ±1.20*	1.60 ±0.22	3.11 ±0.58
(3) Echinaid	7.87 ±1.33 a	5.90 ±1. 54a	2.11 ±0.88	8.45 ±0.13 a	1.28 ±0.22	5.63 ±1.45[a]
(4) *A. fragrantissima* (300 mg/kg)	5.88 ±1.32	4.64 ±0.26 a	1.61 ±0.42	7.66 ±1.18 a	2.41 ±0.42	6.07 ±1.45[a]
(5) *A. fragrantissima* (500 mg/kg)	5.94 ±1.09 a	4.90 ±0.13 a	1.06 ±0.79	7.03 ±1.20 a	2.35 ±0.11	5.17 ±1.26[a]

Means having mark * in the same column have significant values at (P<0.05) compared to the control SRBC group. Means having letter [a]in the same column have significant values at (P<0.05) compared to the Endoxan group challenged with SRBC. Total immunoglobulins (Igs), immunoglobulins M (IgM), immunoglobulins G (IgG). mean ± Standard error (SE)

Table 6: Effect of *Achillea fragrantissima*extract on serum TNF-α, IL-4, and IL-10 cytokines

Groups/ Parameters	TNF-α (pg/ml)	IL-4(pg/ml)	IL-10 (pg/ml)
(1) Control	6.743±1.38	7.491 ±2.64	8.645±1.42
(2) Endoxan	14.651±1.81*	4.063±1.69*	5.432 ±1.59*
(3) Echinaid	8.180±1.77	10.222±1.40[a]	9.675±1.31
(4) *A. fragrantissima* (300 mg/kg)	7.344±3.64	10.554 ±1.22[a]	12.432 ±1.76[a]
(5) *A. fragrantissima* (500 mg/kg)	6.487±1.95	7.830 ±1.71	13.765 ±1.75[a]

Means having mark * in the same column have significant values at (P<0.05) compared to the control SRBC group. Means having letter ain the same column has significant values at (P<0.05) compared to the Endoxangroup challenged with SRBC.Tumor necrosis factor-α (TNF-α), Interlukine-4 (IL-4), and Interlukine-10 (IL-10).mean ± Standard error (SE)

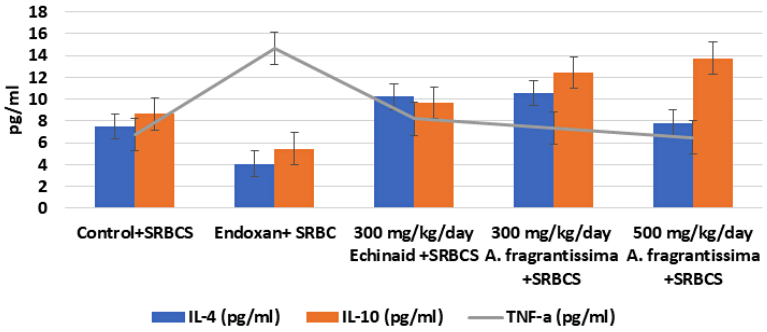

Fig. 1: Combo clustered column line combination chart of TNF-α, IL-4, and IL-10. Sheep erythrocytes (SRBCS), *Achillea fragrantissima* (*A. fragrantissima*).

Spleen Non-Cellular Viability%

Spleen cellular non-viability % values achieved a significant (P≤0.05) increase in group (2) offered Endoxan without plant extract comparable to SRBC control. Whereas there was a significant (P≤0.05) decrease in non-cellular viability % in the ratswho received either Echinaid or 500 mg/kg *A. fragrantissima* extract, they recorded values near to that found in the SRBC control group (7.38±1.12 and 8.12±0.94, vs 8.25±1.25) respectively (Table 7). Nevertheless, *A. fragrantissima* at a low dose (300 mg/kg) showed a non-significant reduction in this parameter.

Scores of The Microscopic Immune Reaction of the Spleen

Regarding the scores of the encountered microscopic immune reaction of the spleen (Table 7), the obtained results signified those rats in the group treated with Endoxan and challenged by SRBC showed lymphoid cell depletion and white pulp atrophy (score of immunosuppression -). Echinaid and *A. fragrantissima* at both doses exerted improvement in the scores of the microscopic immune reaction of the spleen in various degrees. The best improvement recorded in the group received 500 mg/kg *A. fragrantissima* extract which showed prominent lymphoid cell hyperplasia and white pulp hypertrophy (the highest score of immunostimulation ++++) followed by those who received Echinaid that showed moderate lymphoid cell hyperplasia and white pulp hypertrophy (moderate degree of immunostimulation +++) and finally a low dose of *A. fragrantissima* (300 mg/kg) extract which showed mild lymphoid cell hyperplasia and white pulp hypertrophy (mild degree of immunostimulation ++).

Table 7: Effect of *Achillea fragrantissima* extract on splenic non-cellular viability % and microscopic immune reaction score.

Groups/ Parameters	Splenic cellular non-viability%	Splenic microscopical score
(1) Control	8.25±1.25	+
(2) Endoxan	13.97±1.14*	-
(3) *Echinaid*	7.38±1.12[a]	+++
(4) *A. fragrantissima* (300 mg/kg)	7.26±2.14	++
(5) *A. fragrantissima* (500 mg/kg)	8.12±0.94[a]	++++

Means having mark * in the same column have significant values at (P<0.05) compared to the control SRBC group. Means having letter a in the same column have significant values at (P<0.05) compared to the Endoxan group challenged with SRBC. mean ± Standard error (SE). Normal spleen (+), Lymphoid cell depletion (-) Mild lymphoid cell hyperplasia (++), Moderate lymphoid cell hyperplasia (+++), Prominent lymphoid cell hyperplasia (++++).

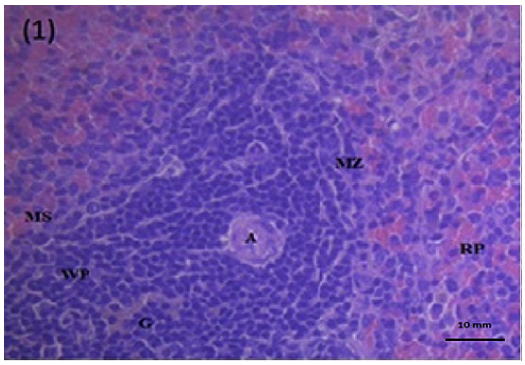

Fig. 2: The Control group revealed mild immunostimulant criteria of the spleen (H&E 40X). MZ: marginal zone; MS: marginal sinus region; G: germinal center; RP: red pulp; WP: white pulp; A: splenic artery

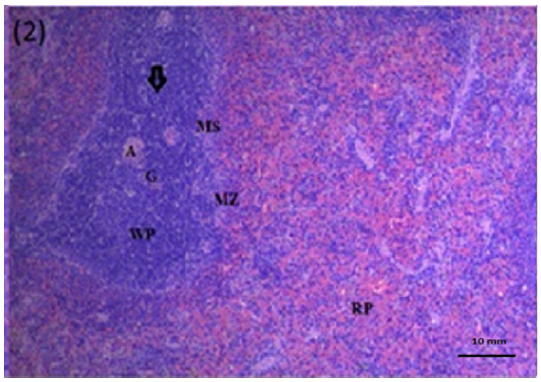

Fig. 3: Endoxan group showed the severest immunosuppressive reaction of the spleen. (H&E 40X). MZ: marginal zone; MS: marginal sinus region; G: germinal center; RP: red pulp; WP: white pulp; A: splenic artery.

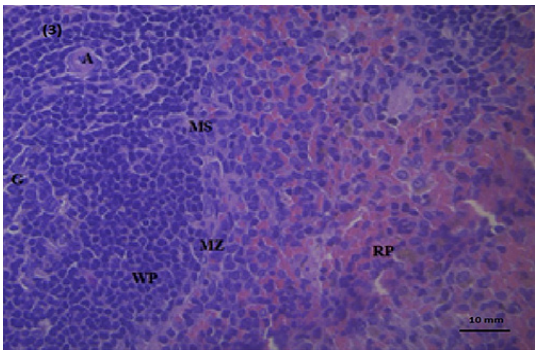

Fig. 4: Echinaid group showed enhancement in the immunostimulant criteria of the spleen, (H&E 40X). MZ: marginal zone; MS: marginal sinus region; G: germinal center; RP: red pulp; WP: white pulp; A: splenic artery.

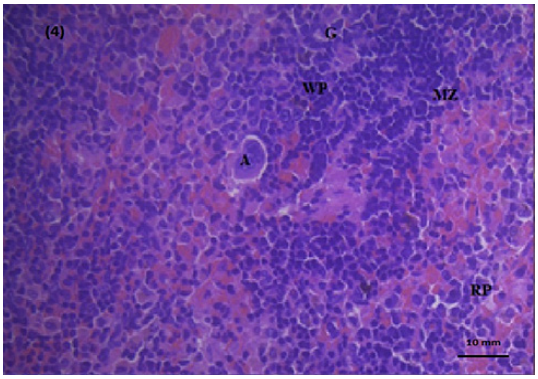

Fig. 5: *Achillea fragrantissima* **(300 mg/kg) group showed improvement in the immunostimulant criteria of the spleen (H&E 40X). MZ: marginal zone; G: germinal center; RP: red pulp; WP: white pulp; A: splenic artery.**

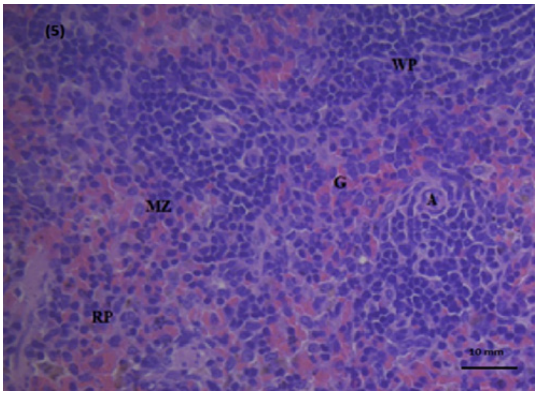

Fig. 6: *Achillea fragrantissima* **(500 mg/kg) group showed more improvement in the immunostimulant criteria of the spleen. (H&E 40X). MZ: marginal zone; G: germinal center; RP: red pulp; WP: white pulp; A: splenic artery.**

Histopathological Observation

The spleen specimens in the control group showed a mild immunostimulant characterized by slight enlargement of the white pulp (WP) and normal red pulp (RP). Endoxan group had an immunosuppressive effect which manifested by severe lymphoid cell necrosis and depletion giving the splenic parenchyma the classic moth-eaten appearance characteristic of immunosuppression besides decreased size and density of WP. Echinaid group showed enhancement of the immunostimulant criteria of the spleen. There were hyperplasia and hypertrophy of lymphoid cells and WP with high lymphocytes respectively. The size and density of G have increased. Administration of *A. fragrantissima* showed improvement of the immunostimulant criteria of the spleen which was clearer with the high dose represented by lymphoid cell hyperplasia and hypertrophy of the WP with high lymphocytes. The hyperplastic lymphoid cells invaded the red pulp zones. The best degree of immunostimulant among all the tested rats was shown in a high dose of *A. fragrantissima* which encountered in both the size and density of the follicular germinal center has increased. the hyperplastic lymphoid cells invaded the RP zones (Fig. 2-6).

Discussion

Most of the results previously published with *A. fragrantissima* extract have been conducted *in vitro*, little information exists *in vivo* and with oral administration. The currentwork was designed to evaluate the immune response of *A. fragrantissima* extract *in vivo* using the SRBC challenge. Highlighting the immunoglobulins and cytokines in comparison with Endoxan and Echinaid as standard immuno-suppressive and immuno-stimulative respectively.

The main bioactive constituents of *A. fragrantissima* obtained in the present study were corresponding with many types of research with some differences in percentage and content. Such variation may be due to some factors, for example, the geographical area that is affected by climate.[36] The major bioactive constituent of *A. fragrantissima* obtained in the present study was camphor as previously recorded[37] The recorded antioxidant results of *A. fragrantissima* confirmed that it has antioxidant properties.[38] Thephenolic compounds have ahigher activity of antioxidants andconfirmed that *A. fragrantissima* has a radical scavenging activity.[39] In any case,the finding indicated that *A. fragrantissima* has a high total phenolic content corresponding to fruits rich in ascorbic acid and other antioxidants.[40]

Endoxan administration had a harmful effect on serum TAC, SOD, and CAT. Moreover, it elevated serum MDA significantly, which is the final product of lipid peroxidation, due to the formation of reactive oxygen species (ROS).[41] The oxidative stress was dramatically increased in mice when received cyclophosphamide.[42] The oxidative stress could be due to metabolic conversion of Endoxan to several types of toxic metabolites, such as acrolein which reacts with cellular nucleophiles, leading to induce the oxidative stress consequence, inhibition of antioxidant enzymes.[43] On the other side, Echinaid extract, and *A. fragrantissima* supplementations significantly alleviated the adverse effect of Endoxan in various manners. They achieved a significant increase in serum SOD, whereas *A. fragrantissima* recorded an improvement in TAC, CAT, and MDA with the dose depending. Administration of ethanol extract of *A. fragrantissima* caused a significant decrease in MDA.[2] Echinaceapurpurea supplementation regulates the activities of MDA, SOD, and CAT levels20.*A. fragrantissima* has biologically active constituents which fight the oxidative stress caused by scavenging of (ROS) and blocking of H2O2-induced mitogen-activated protein kinase (MAPK) pathway.[44] The SOD considers the first-line defense system against reactive oxygen species. It catalyzes to break the two molecules of superoxide into hydrogen peroxide and molecular oxygen so that superoxide becomes less harmful. The CAT enzyme catalyzes the degradation of the hydrogen peroxide to water and oxygen so that completing the detoxification process is initiated by SOD.[45] The antioxidants of the Echinaid and *A. fragrantissima* seem to protect the white blood cells responsible for immunity (neutrophils and lymphocytes) from oxidative stress, preventing their apoptosis.[46] The essential oil of *A. fragrantissima* can be used safely as an antioxidant.[38]

Endoxan administration caused a drop in all antibodies titer measured which were significant for Igs and IgM peri the second dose of SRBC challenge and in Igs and IgG post the second booster dose of SRBC challenge by one week. The obtained

finding might be attributed to cyclophosphamide damaging the DNA of the immune cells, interfering with the proliferation and differentiation of B cells,and subsequence the humoral immune depression occurred.[18] Administration of Echinaid and, *A. fragrantissima* succeeded in rising Igs and IgM titer for the first dose of SRBC and Igs and IgG for the second dose of SRBC significantly. The present results agree with *Mathivanan* and *Kalaiarasi*[47] who concluded that medicinal plants increased antibody titer against SRBC more than virginiamycin. Unfortunately, little research has studied the relationship between *A. fragrantissima* and humoral immunity, whereas many papers studied the effect of the genus Achillea which is all from the same family, *Asteraceae*. Increasing IgG and IgM titer is an indication for enhancement of B lymphocyte production that is involved in the antibody synthesis consequently, improving humoral immune response to SRBCs48. *A. millefolium* L. achieved high IgG and IgM titers against SRBC.[49] In Contrast, inhibition of antibody production was recorded with *A. talagonica* administration.[50] The conflict in the mentioned results is required further studies to solve that dilemma and connect between plant phytochemical constituents and humoral immunity.

Cytokines consider an inflammation regulator and a cornerstone in the pathophysiology of disease inside the human body. One of the immune response measures was depending on the balance between Th1 and Th2.[25] Th1 pro-inflammatory cytokines are represented in the current study by TNF-α. Th2 anti-inflammatory cytokines which represented herein by IL4, and IL.[10] The present results revealed that rats treated with Endoxan and challenged with SRBC showed disturbances in the inflammatory cytokines indicatinga low humoral immune response. These disturbances were reported previously in some studies.[15] Echinaid and *A. fragrantissima* extract mitigated the inflammatory cytokines. There was a reduction in TNF-α, a significant elevation in IL-4, and an increase in IL-10 in the ratswho offer Echinaid. A low dose of *A. fragrantissima* (300 mg/kg) showed a decrease in TNF-α and significant elevation in IL-4 and IL-10. Likewise, a high dose of *A. fragrantissima* (500 mg/kg) caused a modulation in cytokines which was significant in IL-10. The cytokine IL-10 is a key anti-inflammatory regulator ensuring the protection of a host from exaggerated responses to SRBS.[51] The findings are in accordance with many studies that deal with other Achillea species.[52] Few studies were recorded on *A. fragrantissima*, which could cause a significant reduction in proinflammatory cytokines, suggesting a possible cytoprotective effect against cytokines disturbances.[25] Treatment with ethanol and ethyl acetate extracts of *A. fragrantissima* restores the levels of the inflammatory cytokine in the serum to a normal state due to its anti-inflammatory activity.[21] Mostly *A. fragrantissima* extract could inhibit lipopolysaccharide (LPS)-induced expression of TNF-α and downregulated ROS production that leading to its anti-inflammatory effect.[44] In the same context, *A. fragrantissima* extract might be to inhibit Nuclear Factor Kappa B (NFkB) activation subsequence produces pro-inflammation of protein kinase C or p[38] MAPK.[53]

The spleen isan essential organ in mediating the immune status of the body through its filtering of blood-borne pathogens and antigens. The increase in spleen cellular non-viability % caused by Endoxan without plant administration agrees with many types of research.[15,17] Endoxan always has immune-suppressive, oxidative stress effects, and considers a life risk agent.[16] The significant reduction in spleen non-cellular viability % recorded withEchinaid and 500 mg/kg *A. fragrantissima* indicates that Echinaid and *A. fragrantissima* at a high dose success to modulate the immuno suppressive caused with Endoxan. This finding coincided with a recentstudy, which concluded that *A. fragrantissima* extract has an immunostimulant effect concerning humoral and cell-mediated immunity.[54] Echinaid maybe deals with macrophages and T cells by stimulating phagocytosis, and lymphocytic activity to activate the immune response and protect the organism from infection.[20,55]

Histopathological observation of the spleen confirmed the results obtained in an immune response manner. The immune response based upon the score of the microscopic immune reaction of the spleen signified that those rats in the group treated with Endoxan and challenged by SRBC showed lymphoid cell depletion and white pulp atrophy indicatingan immunosuppression effect. This finding may be due to Endoxan causing damage in the spleen and thymus tissues, the main organs in

the immune stimulation.[56] Whereas Echinaid and 500 mg/kg *A. fragrantissima*, exerted prominent lymphoid cell hyperplasia and white pulp hypertrophy ranging from moderate (immunostimulation+++) to high (immunostimulation ++++) scores of the microscopic immune reaction respectively which indicates that *A. fragrantissima* could restore immune response as previously observed.[54]

The beneficial effect of *A. fragrantissima* on immune responses recorded in the current study may be attributed to its antioxidant properties and phytochemical constituents (Camphor, 1,8-Cineole, Artemisia ketone, Thujone, and Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methylene) detected in the present study.

Conclusion

Finally, the results of the current study support the hypothesis that it is possible for individuals who suffer from immunosuppressive, administration of *A. fragrantissima* may be useful as an alternative remedy for prophylaxis for some fatal diseases such as COVID-19 before infection that needs further research to detect the exact dose in humans. Inconclusively the current study highlights that a high dose of *A. fragrantissima* extract is preferred over a low dose to restore and modulate the immune response *in vivo.*

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Conflicts of Interests

The authors declare no conflict of interest in conducting this study.

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