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IMMUNOMODULATORY POTENTIALS OF *RHIPSALIS NEVES-ARMONDII* K. SCHUM. (CACTACEAE) AERIAL PARTS

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Abstract

Rhipsalis neves-armondii K. Schum. (Cactaceae) aerial part preparation has been used for several decades in Nigerian tradetional medicine for treatment of inflammatory disorders and to improve immune function. Little has been reported about the immunomodulatory effects, hence this study was undertaken to evaluate the immunomodulatory potentials of *R. neves-armondii* in rodents.

The aerial succulent part of *R. nerves-armondii* extract (RCE) and fractions (hexane fraction = RHF, ethyl acetate fraction = REF and methanol fraction = RMF) were evaluated for immunomodulatory activity in rodents using delayed-type hypersensitivity response (DTHR), humoral antibody synthesis (HAS), in vivo leukocyte mobilization and in vitro immunostimulatory activity. In addition, acute toxicity, and lethality test (LD_{50}) as well as phytochemical screening tests were also performed.

The RCE (400 mg/kg) and REF (200 mg/kg) elicited 87.9 % significant ($P < 0.01$) increase of the paw edema in DTHR while RHF (400 mg/kg) and RMF (200 mg/kg) elicited 69.7 % and 71.2 % significant ($P < 0.05$) increase of the paw edema in DTHR respectively. The REF (100 mg/kg) significantly ($P < 0.01$) evoked the highest leucocyte mobilization of 211.15 % in a non-dose dependent manner while REF (20 ug/ml) significantly ($P < 0.05$) elicited phagocytic stimulatory activity of 176.36 %. Also, REF demonstrated elevation of antibody titres. Acute toxicity test of RCE showed an estimated value greater than 5000 mg/kg and the phytochemical screening tests for extract and fractions, revealed the presence of notable phytoconstituents like carbohydrates, resins, reducing sugars, alkaloids, terpenoids, flavonoids and steroids.

The extract and fractions of *R. nerves-armondii* exhibited immunostimulatory activities with the ethylacetate fraction (REF) exhibiting the highest activity, an indication that the active phytoconstituent(s) might be soluble ethyl acetate.

Keywords: *antibody, humoral, hypersensitivity reaction, leukocyte, Rhipsalis neves-armondii*

Introduction

Strong body immunity is one of the prerequisites in the fight against several infectious diseases of bacterial and viral origin. Being able to modulate the immune response in order to mitigate diseases is a research area with keen interest [1]. The recent outbreaks of epidemics and pandemic of viral origin have thrown global health into an unprecedented crisis. Worthy of mention is the global threat of COVID-19 pandemic, which resistance to spread among others, is anchored on maintenance of strong body immunity. Both the body humoral and cell mediated immune systems could undergo modulation. Immunomodulatory responses may manifest as immuno-stimulation (potentiation of immune response) and/or immuno-suppression (weakening of immune response). These responses involve regulating immune systems using agents from natural origins, such as plants and animal sources, or synthetic origin, such as standard immune boosters [1]. Since these synthetic agents are associated with serious side effects, unavailability and unaffordability to rural dwellers, alternative approaches, such as use of natural products, are being employed in keeping strong body immunity. Medicinal plants are the bedrock and sources of agents with strong immunomodulatory activities, and many have been shown to possess immunomodulatory activities [2-4].

Folkloric uses of *R. neves-armondii* have been documented and include whooping cough, diabetes, cancer, nose bleeding, rheumatic pains, inflamed wounds [5]. In south-eastern Nigeria, *R. neves-armondii* succulent-aerial-part preparation has been used over the years to manage inflammatory and immune deficient disorders. The morphological characteristics of the plant have been described [6]. Although the anticancer and anti-inflammatory activities of extract and fractions of *R. neves-armondii* has been investigated [7], a lacuna in literature is evident since scientific findings on pharmacological activities of *R. neves-armondii* is limited. Given the challenges posed by the COVID-19 pandemic, immuno-compromised patients and long term adverse effects associated with corticosteroids use, there is the need to identify a new cost-effective, efficacious and possibly safer approach to improve the immune function. Owing to this and in a bid to also justify the traditional use of this plant in improving immune function, the aim of the present study was to evaluate the immunomodulatory properties of *R. neves-armondii* aerial parts.

Methods

Media and chemicals

Dried pulverized R. neves-armondii aerial parts, mechanical milling machine, weighing balance, extraction bottles, filter papers, rotary evaporator, cellophane, electronic balance, micrometer screw guage, bijou bottles, syringes and needles, cannula 18G, silica gel, distilled water, normal saline, phosphate buffered saline, centrifuge, sheep red blood cells, Candida albicans, test tubes, microtitre plates, slides, incubators, Bunsen burner, inoculating rod, autoclave, Sabouraud dextrose agar, Sabouraud dextrose broth (freshly prepared), light microscope, counting chamber, chloroform, EDTA, capillary tubes, Levamisole tablets, cages, markers, methanol, dichloromethane, hexane, ethyl acetate, methanol, Tween 80 and water. All chemicals were of analytical grade.

Animals

Adult Swiss albino rats (100-150 g) and mice (20-30 g) of both sex bred in the Laboratory Animal Facility of the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka (UNN) were used. The rodents were maintained under standard conditions (25±2°C) and were freely fed standard pellets (Guinea feed Nig. LTD) and water. Male sheep was procured from the Faculty of Veterinary Medicine, University of Nigeria, Nsukka and was fed pasture and water. The animals were allowed to acclimatize for 14 days. All animal experiments were in compliance with the National Institute of Health Guide for Care and Use of Laboratory Animals (NIH publication #85-23, revised in 1985). The protocol was approved by the Faculty of Pharmaceutical Sciences Ethical Committee on November 29, 2019 (certification number: DPTFPSUNN-879).

Collection of plant material and preparation of extract

Succulent aerial parts of R. neves-armondii were collected in the month of August. The plant was identified and authenticated by Mr. Alfred O. Ozioko of the International Centre for Ethnomedicines and Drug Development (InterCEDD) Nsukka, Enugu State (voucher number: InterCEDD-1254). The plant name has been checked with http://www.theplantlist.org/ on December 15. 2019. The aerial parts were washed in clean water, cut into small sizes, dried under shade, and milled to coarse powder using a mechanical milling machine. The powdered material (3 kg) was subjected to extraction by cold maceration with methanol and dichloromethane mixture (1:1) for 48 hrs and concentrated in a rotary evaporator under reduced pressure and completely dried over a water bath at 60°C to obtain crude extract (RCE).

Solvent-guided fractionation

The RCE yield (140.35 g) was mixed with silica gel (60-120 mesh size) of equal proportion and triturated in a ceramic mortar to get a uniform mixture. The mixture was then air dried and packed in a silica gel glass column (60 cm in length and 7.5 cm in diameter), for elution with solvents. The solvent-guided fractionation was performed in order of increasing polarity; n-hexane, ethyl acetate, and methanol. Concentrating the solvent fractions by open air evaporation yielded n-hexane (RHF), ethyl acetate (REF) and methanol (RMF) fractions.

Antigen preparation

Sheep blood (10 ml) was withdrawn and introduced into a heparinized tube. Using a centrifuge (set at 3000 rpm), the withdrawn blood in 5 ml of normal saline was washed three times for 10 min each. The washed sheep red blood cells (SRBC) was then used for both challenge and immunization after the concentration was adjusted to 1×10^9 cells/ml using normal saline.

Candida albicans suspension preparation

Typed culture of Candida albicans (ATCC®10231 ™) was obtained from the Department of Pharmaceutical Microbiology and Biotechnology, University of Nigeria, Nsukka. Sabouraud dextrose broth was then used to incubate the *C. albicans* culture overnight. The culture was centrifuged and the upper layer was discarded while the button layer was washed further 4 times with sterile phosphate buffered solution (PBS). This was then suspended in a mixture of rat serum and PBS (4:1). Using 0.5 McFarland standard, the count of *C. albicans* was adjusted to $1x10^9$ cells/ml.

Determination of acute toxicity and LD50

The acute toxicity and lethality (LD50) of RCE was determined in mice using standard procedure as outlined by Akunne and colleagues [7].

Phytochemical screening

Phytochemical tests were evaluated on RCE and fractions using standard procedures as outlined by Akunne and colleagues [7].

Evaluation of delayed type hypersensitivity reaction (DTHR)

The effects of RCE on DTHR was evaluated as earlier described by Ofokansi and colleagues [8] with some modifications. Briefly, rats were divided into 5 groups of 5 animals each (n = 5). Delayed type hypersensitivity was induced in rats using SRBC. The RCE was administered via oral route to the animals. Groups 1, group 2 and group 3 received 100 mg/kg, 200 mg/kg and 400 mg/kg of the extract respectively while group 4 received levamisole, 2.5 mg/kg (positive control) and group 5 received the vehicle (2.5 ml/kg, negative control). The treatment with the extract commenced 3 days before the sensitization and stopped after the challenge. On day zero, 1 hour after administering the extract, 0.02 ml of 10^9 cells/ml of SRBC was given via the subcutaneous (SC) route into the right hind paw of the rats to sensitize them. On day 5, 0.02 ml of 10^9 cells/ml of SRBC was given SC into the left hind paw of the rats to challenge them. The difference in the paw thickness before and 24 hours after the challenge was used to evaluate the edema produced by antigenic challenge in the left hind paw. This was repeated for the different fractions. Percentage inhibition was calculated thus;

Inhibition (%) = (1 - mean of treatment/mean of control) x 100

Evaluation of in vivo leukocyte mobilization test

The effects of RCE on *in vivo* leukocyte mobilization was evaluated as earlier described by Hasson and colleagues [9] with some modifications. Rats were divided into 5 groups of 5 animals (n = 5). Groups 1, group 2 and group 3 received 100 mg/kg, 200 mg/kg and 4000 mg/kg of RCE respectively while group 4 and 5 received levamisole, 2.5 mg/kg (positive control) and the vehicle, 2.5 ml/kg (negative control). One hour after the extract has been administered, the animals received 0.5 ml of 3 % w/v agar suspension in normal saline via intra-peritoneal (IP) route. After 4 hours, the animals were sacrificed while the peritoneum was washed with of 5 % solution of EDTA (5 ml) in Phosphate Buffered Saline (PBS). This was repeated for the various fractions. Total and differential leukocyte counts (TLC and DLC) were performed on the perfusates. This was repeated for the different fractions and the % leukocyte mobilization (PLM) was calculated thus;

PLM (%) = [TLC (Test) – TLC (Control)/TLC (Control)] × 100

Evaluation of in vitro immunostimulatory activity with Candida albicans

To induce clotting, rat blood (0.2 ml) was smeared on sterile glass slide and incubated at 37°C for 20 minutes. The slide was then washed gently with normal saline to prevent washing off neutrophils or polymorphonuclear (PMN) leukocytes adhered to the slides. The slide was then flooded with RCE (20 and 40 ug/ml), the positive control was flooded with Levamisole (20 and 40 ug/ml) while the negative control was flooded with the vehicle and they were then incubated at 37°C for 20 minutes. The slides were then covered with *C. albicans* suspension and incubated at 37°C for 1 hour. The slides were then gently drained and fixed with methanol. Thereafter, they were stained with giemsa stain and phagocytosis was evaluated by viewing with a light microscope under oil immersion (100 X). The Phagocytic Index (PI = number of *C. albicans* cells phagocytosed by PMN leukocytes) was then compared with the PI of the control treatment. This was repeated for the different fractions. Immunostimulation was then calculated thus;

% Phagocytic stimulation (PS) = [PI (test) – PI (control) / PI (control)] – 100

Evaluation of humoral antibody synthesis (HAS)

The effects of RCE on humoral antibody synthesis (HAS) was evaluated as earlier described by Ofokansi and colleagues [8] with some modifications. Rats were divided into 5 groups of 5 animals (n = 5). The RCE was administered via oral route to the animals. Group 1, group 2 and group 3 received 100 mg/kg, 200 mg/kg and 4000 mg/kg body weight of RCE respectively while group 4 received levamisole, 2.5 mg/kg (positive control) and group 5 received the vehicle (2.5 ml/kg, negative control). The treatment commenced 3 days before the sensitization and stopped 5 days after the challenge. On day zero, the animals were sensitized by IP administration of 0.1 ml of the 10^9 cell/ml SRBC. On day 5, the animals were challenged by IP administration of 0.1 ml of the 10^9 cell/ml SRBC. The antibody titre was evaluated before the challenge on day 5 (primary) and secondary titre was evaluated on day 10. A 5 ml blood sample was then withdrawn and the sample was centrifuged and diluted using normal saline in 96-U well microtitre plates to obtain a 25 µl serum. This was then challenged with 25 µl of 1 % v/v SRBC and incubated at 37°C for 1 hour. The antibody titre was determined as haemagglutination arising from the highest dilution. This was repeated for the different fractions.

Statistical analysis

The data obtained were analysed using GraphPad Prism version 8.2.1 and subjected to Dunnett's post hoc test. Results were expressed as Mean ± Standard Error of Mean (S.E.M). Differences between the mean of treated and control groups were calculated at 95 % confidence interval and considered significant at $p < 0.05$ and 0.01.

Results

Extractive yield (%) of RCE and its fractions

The extraction of 3 kg of *R. neves-armondii* aerial parts yielded 200.50 g of RCE. The yield of RCE

calculated as percentage of the starting material was 6.68 % w/w. After fractionation of about 140 g of the RCE, and concentration of the solvent fractions by open air evaporation, the percentage yields were; n-hexane fraction (RHF; 53.7 g and 38.36 % w/w), ethyl acetate fraction (REF; 15.4 g and 10.97 % w/w) and methanol fraction (RMF; 7.4 g and 5.27 % w/w).

Phytochemical analysis

The phytochemical analysis showed presence of the following phytoconstituents; alkaloids, flavonoids, glycosides, steroids, terpenoids and tannins (see table 1). This was in line with the work of Akunne and colleagues [7].

Acute toxicity and LD50 of the extract

The acute toxicity of the RCE showed an estimated LD50 greater than 5000 mg/kg, an indication of high safety profile of the plant [7]. See table 2.

Effect of RCE and its fractions on DTHR

The RCE (400 mg/kg) and REF (200 mg/kg) each significantly ($P < 0.001$) elicited 87.9 % increase of the paw edema while RHF (400 mg/kg) and RMF (200 mg/kg) elicited 69.7 % and 71.2 % increase of the paw edema respectively when compared to the control (see Table 3). The increase in paw edema produced by RCE and REF was better than that elicited by the standard treatment (Levamisole).

Effect of RCE and its fractions on leukocyte mobilisation

At 100 mg/kg, the order of increasing percentage leukocyte mobilization of the extract and fractions was as follows; REF > RMF > RHF > RCE with 211.15, 178.96, 166.42 and 38.57 %, respectively which were significant ($P < 0.05$), while levamisole, a standard agent, exhibited 70.85 % which was non-significant. However, both the extract and fractions exhibited insignificant differential leukocyte count on all the doses tested (see Table 4).

Effect of RCE and its fractions on in vitro immunostimulatory activity

The results showed that both the RCE and REF exhibited dose dependent and significant ($P < 0.05$) percentage phagocytic stimulation with REF showing better activity (see Table 5).

Effect of RCE and its fractions on humoral antibody synthesis

The result indicated that the REF exhibited a better antibody synthesis compared to the control. The REF (100 mg/kg) caused the elevations of primary (2.7±0.34) and secondary (2.73±1.66) SRBC specific antibody titre while 400 mg/kg produced a secondary antibody titre of 4.03±0.69 when compared to the control. The secondary titres when compared to their respective primary titres, were markedly elevated across all dose levels (see Table 6).

Discussion

The RCE and its fractions inhibited DTHR in varying degrees. This was in line with other works reported by some authors on other plants that demonstrated inhibition of DTH in the presence of potent leukocyte mobilization activity [2, 3, 10]. It is known that CD4+ and CD8+ T-cells may trigger DTH [11-13]. Studies have also shown that DTH may arise when there is interaction involving antigen that mediates lymphokines release and antigen-specific T-cells [14, 15]. Also, DTH is a feature of chronic inflammation [3, 16]. Several lines of evidence suggest that DTH reaction is important in host defence. The mechanism behind this elevated delay type hypersensitivity during the cell-mediated immune responses could be due to the sensitized T-lymphocytes [17]. When challenged by the antigen, they are converted to lymphoblasts and secrete a variety of molecules including pro-inflammatory lymphokines, attracting more scavenger cells to the site of reaction [18] and the infiltrating cells are probably immobilized to promote the defensive inflammatory reaction [19]. Thus, RCE and its fractions indicates that they may possess a stimulatory effect on lymphocytes.

All the fractions stimulated leukocyte migration into the periteneal fluid while the neutrophils were more mobilized. This was in line with other works [2, 10, 20, 21]. However, this varied with works on other plants which showed a dose dependent increase in leukocyte mobilization [3]. In phagocytosis, the presence of foreign body causing neutrophils to move towards it is an

important step [22]. It has been suggested that levamisole can improve immune function [23]. Circulating leukocytes migrate to the injured site and assist in tissue repair [24]. Also, antigens, activated T-cells may facilitate recruitment of leukocytes [24]. Tissue sentinel cells would induce the release of pro-inflammatory mediators thereby stimulating leukocyte recruitment [24]. One of the vital steps in leukocyte migration involves the initiation of weak adhesive interactions between leukocytes and endothelial cells within the inflamed tissues [24]. This aids in the activation of leukocytes on the luminal part of the blood vessels, thereby facilitating firm leukocyte adhesion [24]. These principles govern the route of leukocyte migration along the endothelial cell barrier. Trans-migrated leukocytes show enhanced survival, and increased efficiency in killing and clearing pathogens. Consequently, breaching of venular walls would stongly aid in facilitating leukocyte migration into inflamed tissues and would also acts as a key process through which tissue-infiltrated leukocytes are primed for delivering an effective immune response [25, 26]. The importance of this action is that *R. neves-armondii* may likely quicken cellular response to inflammation and clearly indicates its immunostimulating properties.

The extract and fractions of *R. neves-armondii* caused phagocytic stimulations. This indicates that the extract and fraction stimulated the PMN leukocyte cells leading to engulfing of the *Candida* cells [27].

The fractions (REF) stimulated an increase in antibody titres. This slightly varied with results of works on different plants that possessed leukocyte mobilization activity [2, 3]. It has been suggested that humoral mediated immunity may require the development of immunoglobulins by specific antigen sensitization [28]. Antibody synthesis require co-operation of macrophages, B-lymphocytes and T-lymphocytes [29, 30]. Antibody molecules, a product of B-lymphocytes and plasma cells, are central to humoral immune responses. Immunoglobulin G and Immunoglobulin M are the major immunoglobulins which are involved in the complement activation, opsonisation and neutralization of toxin. A potent immunostimulant should be able to stimulate sufficient production of antibodies to help fight infection. This activity may improve humoral immune function which could lead to lyses and death of the antigenic cells [31]. Thus, *R. neves-armondii* possesses immunostimulatory activity.

The extract and fractions of *R. neves-armondii* gave positive reactions several phytochemicals. It has been observed that flavonoids may induce the proliferation of peripheral blood leukocyte in humans [32, 33]. Some studies have also suggested that flavonoids may induce an elevation in interferon, helper T-cells and macrophages thus helpful in immune dysfunction [34-36]. The presence of these phytoconstituents could be responsible for the immunomodulatory activity of RCE and its fractions (though no specific phytochemical may be attributed to its immunomodulatory activity at the stage of this work).

Conclusion

The extract and fractions of *R. neves-armondii* succulent aerial parts possess immunomodulatory properties via stimulation of both humoral and cell mediated immune functions, with ethyl acetate fraction as the most active fraction. These findings, therefore, support the traditional claim in using the plant for immune boosting properties. Further work is recommended towards the isolation and characterization of the phytoconstituent(s) responsible for the immunostimulatory properties.

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Table 1: Phytoconstituents of *R. neves-armondii* aerial part *extract* and fractions

Constituents	RCE	RHF	REF
Alkaloids	+	-	+
Carbohydrates	+	+	-
Flavonoids	+	+	+
Fats and Oil	-	-	-
Glycosides	-	-	-
Reducing sugars	+	+	-
Resins	+	+	+
Saponins	-	-	-
Steroids	+	+	+
Tannins	-	-	-
Terpenoids	+	+	+

RCE = crude extract, RHF = hexane fraction, REF = ethyl acetae fraction, + = present, - = absent

Table 2: Acute toxicity and LD50 of *R. neves-armondii* aerial part extract

1st phase (24 hours)		2nd phase (24 hours)	
Dose (mg/kg)	Mortality	Dose (mg/kg)	Mortality
10	0/3	1600	0/1
100	0/3	2900	0/1
1000	0/3	5000	0/1

RCE = crude extract

Table 3: Effect of *R. neves-armondii* aerial part *extract* and fractions on DTHR

Treatment	Dose (mg/kg)	DTH (mm)	% increase in paw edema
RCE	100	0.22±0.05*	66.0
	200	0.42±0.13	37.9
	400	0.08±0.06**	87.9
RHF	100	0.30±0.17*	55.0
	200	0.47±0.10	28.9
	400	0.20±0.07*	69.7
REF	100	0.25±0.09*	62.0
	200	0.08±0.07**	87.9
	400	0.26±0.07*	60.6
RMF	100	0.26±0.07*	60.6
	200	0.19±0.09*	71.2
	400	0.22±0.10*	66.6
Levamisole	2.5	0.21±0.09	68.2
Control	2.5 ml/kg	0.66±0.06	-

**P < 0.05, **P < 0.01* compared to control, n = 5, values of DTH are presented as mean±SEM, DTH = delayed type hypersensitivity, RCE = crude extract, RHF = hexane fraction, RMF = methanol fraction, REF = ethyl acetate fraction,

Table 4: Effect of *R. neves-armondii* aerial part *extract* and fractions on leukocyte mobilisation

Treatment	Dose (mg/kg)	TLC (cells/mm3)	LM (%)	DLC	
				Neu	Lym
RCE	100	3344.2±249.83	38.57	76.1±0.81	24.8±0.81
	200	2743.4±216.40	13.67	69.0±1.22	31.0±1.22
	400	2333.9±298.43	3.29	63.4±1.32	36.6±1.32
RHF	100	6430.0±437.63	166.42*	70.5±0.64	29.5±0.64
	200	7039.2±470.69	191.67*	63.2±0.69	36.7±0.73
	400	7117.0±193.65	194.89*	61.6±0.97	38.5±1.70
REF	100	7509.4±418.26	211.15**	69.4±2.17	30.6±2.17
	200	6923.8±350.45	186.89*	67.7±2.96	32.2±3.02
	400	6473.2±328.45	168.22*	59.4±1.39	40.6±1.39
RMF	100	6732.4±482.02	178.96*	68.1±0.06	31.8±1.10
	200	6188.6±313.84	156.43*	66.1±2.17	33.9±2.17
	400	4898.0±404.10	102.95*	60.0±2.02	39.2±1.87
Lev	2.5	4123.4±409.04	70.85	74.0±0.94	26.0±0.94
Control	2.5 ml/kg	2414.4±143.77	-	69.2±0.37	30.8±0.37

**P < 0.05, **P < 0.01* compared to control, n = 5, values of TLC and DLC are presented as mean±SEM, RCE = crude extract, RHF = hexane fraction, REF = ethyl acetate fraction, RMF = methanol fraction, lev = levamisole, TLC = total leukocyte count, LM = leukocyte mobilization, DLC = differential leukocyte count, Neu = neutrophils, Lym = lymphocytes

Table 5: Effect of *R. neves-armondii* aerial part *extract* and ethyl acetate fraction on *in vitro* immunostimulatory activity

Treatment	Dose (ug/ml)	PI (cells/mm3)	% Phagocytic Stimulation
RCE	40	70	153.55*
	20	52	89.09
REF	40	76	176.36*
	20	58	110.90*
Levamisole	40	74	169.09*
	20	66	140.00*
Control	-	28	-

**P < 0.05* compared to control, n = 5, RCE = crude extract, REF = ethyl acetate fraction, PI = Phagocytic Index

Table 6: Effect of *R. neves-armondii* aerial part ethyl acetate fraction on humoral antibody synthesis

Treatment	Dose (mg/kg)	HAS (mean titre±SEM)	
		primary	secondary
REF	100	2.70±0.34	2.73±1.66
	200	2.00±1.11	2.15±1.99
	400	1.64±0.45	4.03±0.69
Levamisole	2.5	2.53±0.08	3.97±0.70
Control	2.5 ml/kg	2.15±0.20	3.05±0.79

n = 5, values of HAS are presented as mean±SEM, REF = ethyl acetate fraction, HAS = humoral antibody synthesis