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## Research Article

# Supercritical Carbon Dioxide Extract of Seabuckthorn Leaves Enhances Rabies Virus Neutralizing Antibody Titers and CTL Response in Swiss Albino Mice

### Abstract

**Introduction:** Rabies is a viral disease that causes nearly thousands of death globally per year. Vaccination against rabies generates virus neutralizing antibodies and is the most successful and cost effective method of preventing the disease. In the present study, we have evaluated the adjuvant property of supercritical carbon dioxide extract (SCE) 300ET of Seabuckthorn (SBT) leaves against inactivated rabies virus antigen in Swiss albino mice.

**Methods:** Mice were grouped as PBS control; inactivated rabies antigen (Rb) control; 300ET+Rb and Algel+Rb. All the mice were primed on day 1 followed by single booster at day 14. Sera were collected at different time points for RVNA analysis. Additionally, the effect of SCE on CD8<sup>+</sup>Granzyme B<sup>+</sup> Cytotoxic T Lymphocyte (CTL) response, surface markers and cytokine levels were measured.

**Result:** The results showed enhanced rabies virus neutralizing antibody (RVNA) titres using 300ET as compared to control and Algel groups as measured by Rapid Fluorescent Focus Inhibition Test (RFFIT). Moreover, the extract 300ET and Algel showed increased CD138<sup>+</sup> (plasma cells) and CD11c<sup>+</sup> (dendritic cells) cell population as compared to the inactivated rabies antigen immunized mice. The extract 300ET also stimulated cellular immunity by showing heightened CTL response and proinflammatory cytokines level such as TNF- $\alpha$  and IL-1 $\beta$ .

**Conclusion:** Hence, our results suggested that SCE300ET exhibits adjuvant activity by effectively enhancing antibody as well as cell mediated immunity in response to inactivated rabies antigen. Thus, SCE could be considered as a potential adjuvant candidate for rabies vaccines.

## Abbreviations

SBT: Seabuckthorn; RVNA: Rabies Virus Neutralizing Antibody; RFFIT: Rapid Fluorescent Focus Inhibition Test; LPS: Lipopolysaccharide; MPL A: Monophosphoryl Lipid A; CTL: Cytotoxic T-lymphocyte; CFTRI: Central Food Technological Research Institute; SC-CO<sub>2</sub>: Supercritical Carbon Dioxide; SCE: Supercritical Carbon Dioxide Extract; Rb: Inactivated Rabies antigen; QS21: *Quillaja saponaria* fraction 21; TNF- $\alpha$ : Tumor Necrosis Factor-alpha; IL-1 $\beta$ : InterLeukin-1 beta; CD: Cluster of Differentiation; FBS: Fetal Bovine Serum; PE: Phycoerythrin; FITC: Fluorescein IsoThio Cyanate.

## Introduction

Vaccines stimulate strong immune response against various pathogens and is the most successful and cost effective measure for the prevention of several infectious diseases [1]. However, new generation vaccines consist of purified, recombinant or synthetic subunits which provide safety but often result in weaker immunogenicity [2]. Therefore, due to weaker immunogenicity, such vaccine formulations often require the use of adjuvants.

Adjuvants (from Latin word-*adjuvare* meaning to *help*) are the immune stimulants that when used in combination with vaccine antigens, enhance specific or non-specific immune responses [3]. Several adjuvants have been developed and some such as Alum, MF59 and monophosphoryl lipid A (MPL A) have got licensed for use in human vaccines. For more than 90 years, Alum has been the only adjuvant widely used in human vaccines [4]. The downsides of alum as a vaccine adjuvant includes its incapability to induce cell mediated immune responses [5,6]. Therefore, there is an urgent need to develop an adjuvant that is safe and stimulate both humoral and cellular arms of immune system.

Plants and their derivatives are very well known immunomodulators and are the major source of traditional medicines used for the prevention of various infectious diseases [7]. There are several studies reporting the adjuvant activity of plant extracts [8-10] and the most extensively studied plant based adjuvant is QS21, a saponin isolated from the bark of *Quillaja saponaria* tree. Despite undesirable side effects [11,12], QS21 exhibits adjuvant activity by inducing both humoral and cell mediated immune response against various vaccine antigens [13]. Therefore, to develop an adjuvant with maximum immunogenicity and no toxicity remains a major challenge and an unmet need.

The present study involves evaluation of the adjuvant activity of supercritical carbon dioxide extract (SCE) from the leaves of Seabuckthorn (SBT) (*Hippophae rhamnoides* L.) in response to inactivated rabies antigen. *Hippophae rhamnoides* L. is a high altitude medicinal plant, belongs to the family Elaeagnaceae and has been reported to have several pharmacological and nutritional properties such as immunomodulatory, anti-stress, anti-inflammatory, tissue regeneration, anti-oxidant, anti-aging, anti-microbial etc [14-19]. In this study, we have investigated the adjuvant activity of SCE with inactivated rabies virus antigen and showed that the SCE induced both humoral and cell-mediated immune responses against inactivated rabies virus antigen.

## Materials and Methods

### Antigen

Inactivated rabies virus (CVS-BHK strain) antigen and Algel were obtained from Indian Immunological Ltd., Hyderabad, India.

### Collection of plant material

Seabuckthorn leaves were collected from hilly regions of Western Himalyas, India, in the month of September, when the plant grows widely under natural conditions. The whole leaves were washed thrice with distilled water and then shade dried at temperature  $36 \pm 1$  °C and humidity 32% for 2-3 weeks in a clean dust free environment for extraction procedure. Voucher specimen (058/FRL/2002) is preserved at Defence Institute of High Altitude Research, DRDO, Leh, India, after Ethno-botanical identification of species.

### Preparation of extract by Supercritical Carbon Dioxide (SC-CO<sub>2</sub>) Extraction procedure

A pilot scale High-pressure SC-CO<sub>2</sub> extractor (NOVA Swiss WERKE AG, EX 1000-1.4-1.2 type, Switzerland), designed to working pressures of up to 1000 bar and temperature up to 100°C, installed at the Department of Food Engineering, CFTRI, Mysore, was used for the experimental work. The SCE was prepared first by soaking the dried and powdered SBT leaves in ethanol (5 : 1 w/v) overnight at room temperature ( $25 \pm 1$ °C) for equilibration. The process was repeated thrice for complete extraction. The dried powdered SBT leaves were extracted at 300 bar pressure and 60°C temperature in the presence of ethanol (co-solvent), used as entrainer during extraction. Entrainers aid in extracting polar compounds by modifying the solubility of supercritical fluid. Solvent was removed and the dried sample was stored at 4°C for further analysis. The stock concentration of SCE prepared for *in-vivo* studies was 0.1 mg/ml and the dose optimized after screening studies (Data not shown) was 0.1µg/100µl/mouse.

### Experimental animals

Healthy, male/female, 8-12 week old Swiss albino mice weighing 25-30 g, were obtained from the Experimental Animal Facility at DIPAS for the study. The animals were maintained under controlled environment in the Institute's animal house with food and water *ad libitum*. All the experiments were performed in accordance with the regulations specified by the Institute's Animal Ethical Committee and conform to the national guidelines on the care and use of laboratory animals, India.

## Immunization

Mice were intraperitoneally (i/p.) administered with SCE300ET or intramuscularly (i.m.) with Algel (5% v/v of the vaccine formulation) mixed with inactivated rabies antigen (5% v/v). All the animals were immunized with the final volume of 100 µl of formulation. The control groups included mice immunized with PBS and rabies antigen alone. Pre-immune sera were collected from all the mice on day zero. Mice were primed on day one followed by a booster dose on day 14. Sera samples were collected on days 7, 14, 21, 28 and 35. Sera collected on days 7 & 14 and days 21 & 28 were pooled and stored at -80 °C for analyzing rabies virus neutralizing antibody (RVNA) titres. Experiments were repeated thrice.

### Estimation of rabies virus neutralising antibody (RVNA) titres

RVNA titres were measured by Rapid Fluorescent Focus inhibition test (RFFIT) as per WHO recommended procedure [20], with some modifications. We used BHK 21 (ATCC CCL 10) and BHK21 adapted CVS 13 strain of rabies virus. The reference serum used was an inhouse serum calibrated against 2<sup>nd</sup> International reference standard (National Institute of Biological standards, UK) having a titer of 30 IU/mL. Briefly, doubling dilutions of serum samples and reference serum (after heat inactivation at 56°C for 30 min in a water bath) in duplicate were made in 96 well plates using Iscove's Modified Dulbecco's Medium (IMDM) (Sigma). To each 100 µl of serum dilution 100 µl of CVS (100 FFD<sub>50</sub>) was added and the plate was incubated at 37°C for 1 h. A confluent monolayer of BHK 21 cells were trypsinized and resuspended in 10 ml of IMDM with 10% FCS (Sigma). Cell control and virus controls were also included. To each well of the 96 well plate 100 µl of cell suspension was added and the plate was incubated at 37 °C in a CO<sub>2</sub> incubator (Sanyo, Japan). After 24 h the cells were fixed in cold acetone for 30 minutes and stained by direct FAT using commercially available rabies N conjugate. (Light diagnostics, USA). The plates were then observed under an inverted fluorescence microscope (Nikon Eclipse, Japan). The highest dilution of serum showing 50% inhibition of fluorescence foci was taken as end point dilution. The titer was converted to IU/mL in comparison with reference serum.

### Cytotoxic lymphocyte activity

Heparinised blood was collected by retro-orbital puncture four to five days post booster from immunized mice to evaluate the effect of SCE300ET on CD8<sup>+</sup> Granzyme B<sup>+</sup> CTLs. Briefly, RBCs were lysed using FACS lysing solution, washed twice with PBS followed by the staining of the cells with FITC labelled CD8 for 30 min. Cells were washed with PBS, fixed in Cytofix (BD Biosciences), permeabilized using Cytoperm (BD Biosciences) and labelled with PE conjugated Granzyme B antibody. Stained cell population was counted using FACS Calibur Flowcytometer (BD Biosciences, USA).

### Estimation of TNF-alpha and IL-1beta in peritoneal macrophages

The cytokine concentrations were evaluated in the culture supernatants of LPS (Sigma) stimulated peritoneal macrophages using commercially available ELISA kits following manufacturer's

instructions (ebiosciences). Briefly, intra-peritoneal injection of thioglycollate (10% w/v) was given after administration of booster on day 14. Mice were euthanized after 72 h using chloroform. Peritoneal macrophages from immunized mice were aseptically isolated as described earlier (Jayashankar et al., 2012). The cell suspension was transferred in ice-cold sterile conical tubes and centrifuged at 1500 rpm for eight minutes. Supernatant was discarded and the cells ( $1 \times 10^6$  cells  $\text{ml}^{-1}$ ) were suspended in RPMI supplemented with 10% FBS. The cells were cultured in 96 wells plate in the presence of LPS ( $1 \mu\text{g}/\text{ml}$ ) for 48 h in 5%  $\text{CO}_2$  incubator at  $37^\circ\text{C}$  for the analysis of TNF- $\alpha$  and IL-1 $\beta$  cytokines production. Cell culture supernatants were harvested after the incubation and stored at  $-80^\circ\text{C}$  until used.

### Expression of CD138 in whole blood

Whole blood from the immunized animals were collected in separate heparinized tubes and stained with anti-mouse PE labelled CD138 antibody for forty-five minutes in dark at room temperature. Red blood cells were lysed by BD FACS lysing solution and washed twice with 1ml of 1X PBS. Finally, cells were suspended in 500 $\mu\text{l}$  PBS and samples were analyzed on FACS Calibur (Becton Dickinson, San Jose, CA, USA) using Cell Quest Pro software.

### Expression of CD11c in peritoneal lavage

Mouse peritoneal cells ( $1 \times 10^6$  cells) isolated from each group were suspended in 200 $\mu\text{l}$  PBS. The cells were stained with anti-mouse CD11c-FITC labelled antibody for 45 minutes in dark at room temperature. Finally, cells were washed once with 1ml of 1X PBS and suspended in 500 $\mu\text{l}$  PBS. The samples were then acquired and analyzed on a FACS Calibur (Becton Dickinson, San Jose, CA, USA) using Cell Quest Pro software.

### Statistical analysis

SPSS 15.0 software was used for calculating statistical significance by applying two tailed Student's *t*-test and one way Anova wherever applicable for comparing the control groups with the treated groups. Differences were considered significant at  $p < 0.05$ .

## Results

### Estimation of rabies neutralizing antibody titres

There was enhanced rabies virus neutralizing antibody (RVNA) titres observed by RFFIT in pooled sera samples of mice immunized with SCE300ET+Rb and Algel+Rb from the 7<sup>th</sup> & 14<sup>th</sup> and 21<sup>st</sup> & 28<sup>th</sup> and 35<sup>th</sup> day (Figure 1) as compared to mice immunized with PBS control ( $p \leq 0.01$  and  $p < 0.05$  vs PBS). SCE300ET+Rb ( $13.6 \pm 2.89$  IU/ml) and Algel+Rb ( $12.35 \pm 3.91$  IU/ml) showed higher RVNA titres in 21<sup>st</sup> & 28<sup>th</sup> day pooled sera samples ( $p < 0.01$ ,  $p < 0.05$  vs Rb) than the Rb immunized group (0 IU/ml). Interestingly, 300ET+Rb ( $p \leq 0.01$  vs Algel+Rb) group showed significant increase in RVNA titres as compared to Algel+Rb group.

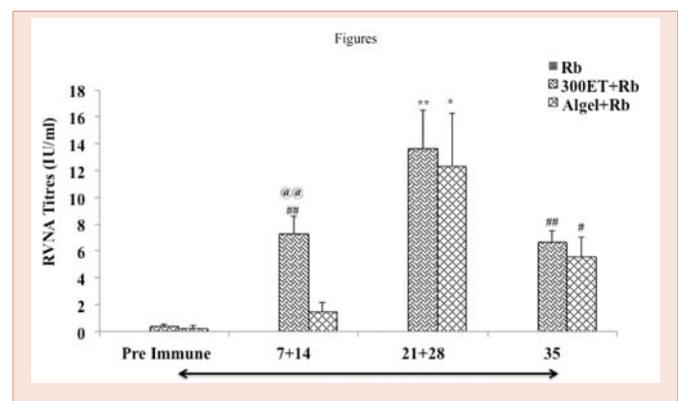
### CTL activity

The CTL activity of SCE300ET against inactivated Rb antigen was performed by measuring CD8<sup>+</sup> Granzyme B<sup>+</sup> population in peripheral blood of immunized mice by Flowcytometry (Figure 2). The

extract 300ET+Rb ( $5.67 \pm 0.98$  %) immunized mice showed highest percentage of CD8<sup>+</sup> Granzyme B<sup>+</sup> cell population as compared to the Algel-rabies immunized mice ( $3.23 \pm 1.11$  %) or with rabies antigen alone ( $1.59 \pm 0.04$  %).

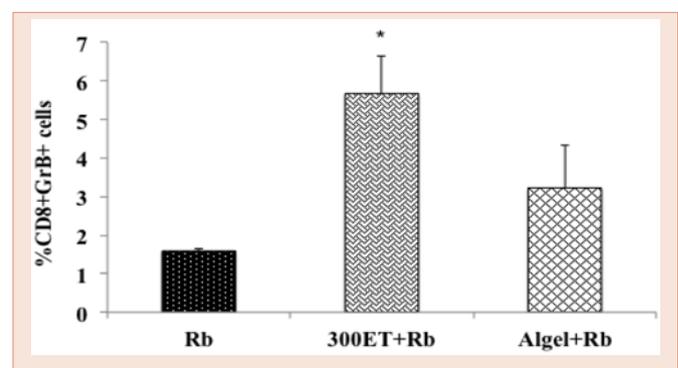
### Effect of SCE300ET on TNF-alpha and IL-1beta in peritoneal macrophages

The LPS induced cytokines TNF- $\alpha$  and IL-1 $\beta$  were measured in the supernatants of cultured peritoneal macrophages from immunized mice and were found to be significantly enhanced ( $p \leq 0.01$  and  $p < 0.05$  vs Rb) in the 300ET+Rb treated group ( $1787.09 \pm 217.12$  pg/ml;  $1803 \pm 25.98$  pg/ml) as compared to the Rb alone ( $1188.89 \pm 97.58$  pg/ml;  $1578.71 \pm 16.08$  pg/ml) administered group (Figure 3A,3B).



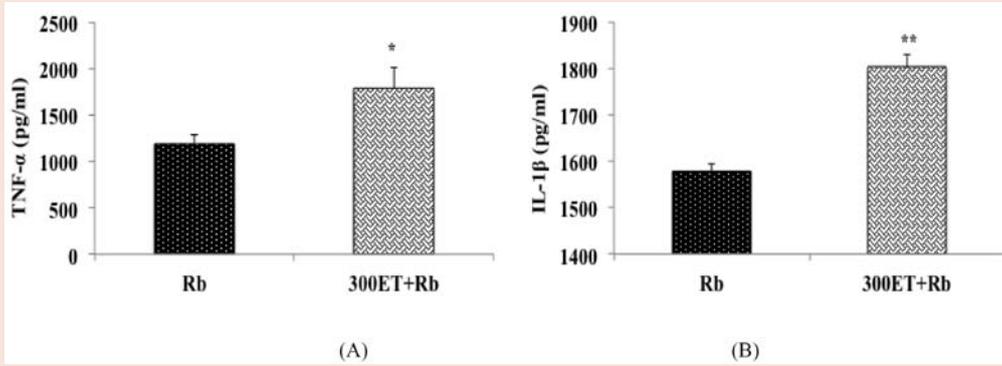
**Figure 1:** Rabies virus neutralizing antibody titres in mice sera.

Figure 1, Effect of SCE on RVNA titres: The figure depicts the production of RVNA titres in immunized mice sera samples collected on Day 0 (Pre-immunization); Days 7 and 14 (pooled); Days 21 and 28 (pooled) and Day 35. Mice were grouped as PBS (n=8); Rb control (n=5), 300ET+Rb (n=6-10), and Algel+Rb (n=10). Booster was administered on day 14. One way Anova was applied to determine the statistical significance between the control and treatment groups  $p < 0.05$  was considered to be significant (## $p \leq 0.01$ , \* $p < 0.05$  vs PBS; \*\* $p < 0.01$  \* $p < 0.05$  vs Rb; @@ $p \leq 0.01$  vs Algel+Rb). Protective neutralization antibody titre limit was considered  $< 0.5$  IU/ml. PBS: Phosphate Buffered Saline; Rb: Inactivated Rabies antigen; RVNA: Rabies Virus Neutralizing Antibody.



**Figure 2:** CTL response by SCE300ET.

Figure 2, Effect of SCE300ET on CTL Activity: The figure shows the percent CD8<sup>+</sup> GrB<sup>+</sup> cell population of different groups. Rb: Animals immunized with inactivated rabies antigen; 300ET+Rb: Animals immunized with the formulation containing SCE300ET and Rb antigen; Algel+Rb: Animals immunized with the formulation containing Algel and Rb antigen. The graph represents mean  $\pm$  SEM (n=3). Statistical significance was shown as \* $p < 0.05$  vs Rb.



**Figure 3:** Effect on Cytokines level by SCE300ET.

Figure 3, Effect of SCE300ET on LPS induced (A) TNF-α and (B) IL-1β cytokines level in response to inactivated rabies antigen. The levels of TNF-α and IL-1β cytokines were measured in the supernatants of peritoneal macrophages of immunized mice stimulated with LPS for 48h. The bar graph represents the mean ± SEM (n=3). Statistical significance was shown as \*p<0.05, \*\*p<0.01 vs Rb.

### Effect of SCE300ET on CD138 and CD11c expression

The whole blood and peritoneal cells from individual mice from different groups were stained with flourochrome conjugated anti mouse CD138 and CD11c respectively. The expression of both CD138 and CD11c were enhanced significantly in 300ET+Rb (20.18±2.97 %; 25.13±3.37 %) and Algel+Rb (20.83±3.58 %; 25.84±1.62 %) immunized groups as compared to the Rb antigen alone (9.56±1.88 %; 4.85±1.26 %) immunized group (Figure 4A,C,4B,D).

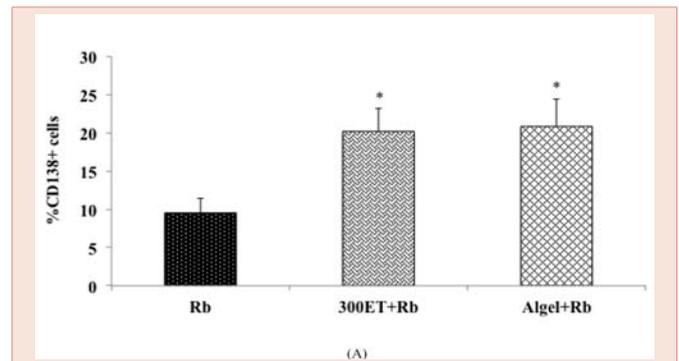
### Discussion

Plant extracts with strong immunomodulatory properties are being evaluated for their adjuvant activity and therefore, are of great importance particularly in developing new adjuvants. There are several studies reporting the adjuvant potential of crude plant extracts [3,10] and their derivatives such as polysaccharides, saponins etc [21-23], stimulating both humoral and cell mediated arms of immunity.

In this study, we have reported that the extract 300ET exhibits adjuvant activity by augmenting both humoral as well as cell mediated immune responses against inactivated rabies virus antigen. The extract 300ET plus Rb antigen administered group has shown heightened RVNA titres, increased CTL and pro inflammatory cytokines production. Also, we observed that the extract 300ET has shown better immunity than Algel in response to inactivated rabies antigen.

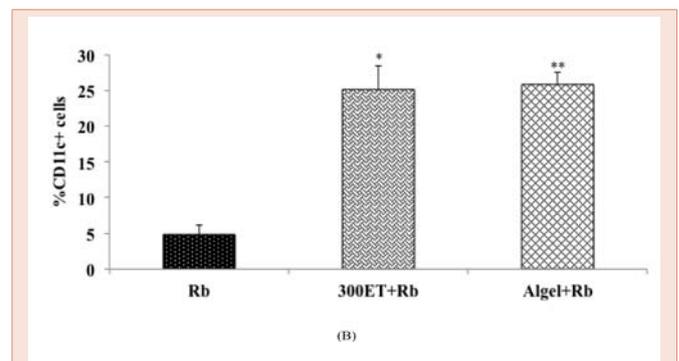
The adjuvant properties of different compounds are being evaluated successfully using different experimental animals (including mice, rats etc) and the primary goal is to obtain higher antibody titers with maximum affinity, in order to enhance antigen specific response in both intensity and duration. Vaccination against viral infections confers protection by generating neutralizing antibodies [24]. Since antibody production is crucial for the prevention of viral infections, we have shown that SCE300ET when administered along with Rb antigen, significantly enhanced the RVNA titres and the antibody titers were found to be higher as compared to Algel and rabies antigen immunized mice. CD138 is a marker for plasma cells (PCs) or

antibody producing cells [25], that are differentiated from the antigen activated B cells [26]. In the present study, the increase in antibody titers was further confirmed by the increased expression of CD138+ cells in whole blood of 300ET+Rb and Algel+Rb immunized mice as compared to the inactivated Rb immunized mice.



**Figure 4a:** Increased expression of surface markers by SCE300ET.

Figure 4, Effect of SCE300ET on surface markers: The figures (A) & (B) represent the percent cells while (C) & (D) are the representative dot-plots of CD138+ plasma cells and CD11c+ cells in the whole blood and peritoneal lavage respectively. The bar graph represents mean ± SEM (n=3). Statistical significance was shown as \*p<0.05, \*\*p<0.01 vs Rb.



**Figure 4b:**

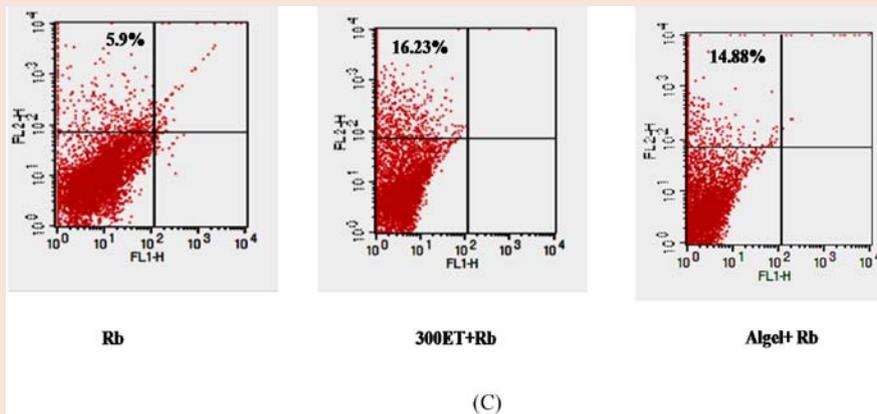


Figure 4c:

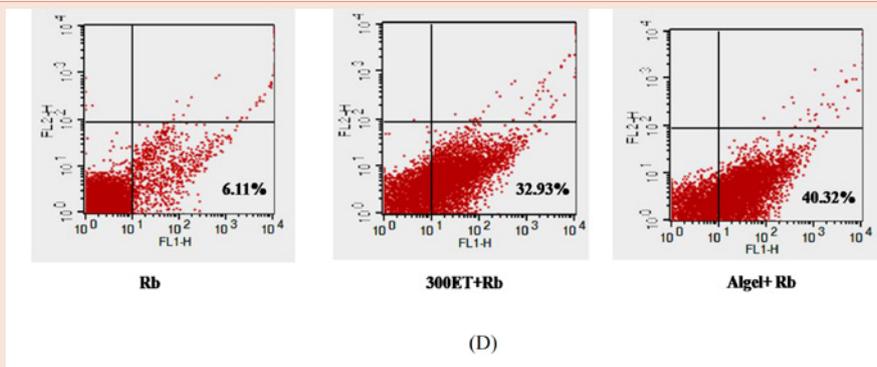


Figure 4d:

Since antibodies generated against viruses can only neutralize or block viruses rather than killing them, one of the most effective mechanisms that facilitate clearance of viruses is mediated by CTLs (CD8<sup>+</sup> T lymphocytes). CTLs are activated by stimuli like antigens or pathogens and these activated CTLs subsequently kill infected or damaged cells by secreting cytolytic mediators, perforin or granzymes [27]. Therefore, in our study we have also shown the effect of SCE300ET on CTLs in response to rabies virus antigen and found that the CD8<sup>+</sup>Granzyme B<sup>+</sup> CTL cells was higher in 300ET-rabies immunized group than Rb and Algel+Rb immunized group.

Cytokines play an important role in modulating immune responses to viral infections [28]. Schijns and colleagues [28] reported that vaccine induced antibody responses against inactivated rabies antigen can be increased by administering cytokines like TNF- $\alpha$ , IL-1, IL-2 and IFN- $\gamma$  and thus, improves the vaccine efficacy. Besides, Faber and colleagues showed that TNF- $\alpha$  exhibits antiviral activity against rabies virus infection by inducing inflammatory response [29]. In our study also, we report that the extract 300ET enhanced inflammatory cytokine production like TNF- $\alpha$  and IL-1 beta that could possibly aid in viral clearance.

Dendritic cells (DCs) are professional antigen presenting cells (APCs) that play key role in inducing protective immune responses

to bacterial or viral infections. Wen et al., reported that increased expression of CD11c positive DCs is essential in inducing protective immunity in response to rabies virus [30]. In this study, we have also shown increased expression of peritoneal CD11c<sup>+</sup> cells in the extract 300ET+Rb immunized group as compared to Rb alone immunized group.

## Conclusion

Based on these findings, our study demonstrates that SCE300ET enhanced both humoral and cellular immunity in response to inactivated rabies virus antigen in mice. Hence, the present work suggests that the supercritical extract 300ET has strong immunomodulatory activity and suitable as a promising adjuvant candidate for rabies vaccines.

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