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Original Research

Effect of ultra-diluted ethanol extract of Rhus toxicodendron SARS-CoV-2 Spike protein RBD induced inflammation in chick embryo

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Abstract

Rhus Toxicodendron (RT) extract has been used in homeopathic and phytomedicines for a few centuries. RT chiefly comprises a bioactive chemical named Urushiol. Studies showed RT extracts have potential immunomodulatory, anti-inflammatory, and anti-arthritic activities. In this experiment, we studied whether ethanolic extracts of RT can inhibit Delta SARS-Cov-2 spike protein RBD-induced inflammation, leading to cytokine inequity in fertilized chick embryos (Gallus gallus domesticus). Inoculation of Delta SARS-CoV2 spike RBD protein was done in 14th-day-old chick embryos along with control, pre-, and post-treatment sets consisting of ultra-diluted RT extract. Allantoic fluids from the eggs were collected and preserved at -80 ºC after 48 h to study different cytokines. Dissect ion was done, and the liver of each animal was collected and sent for histological study. The most prominent result was up-regulation in the expression of Interferon alpha and Interleukin-10 genes in RT 6 CH challenged, pre-treatment, and post-treatment experimental sets. However, IL 6, IL 8, IL1B, IFN-beta, IFN-gamma, and TGF beta expressions were insignificant in all the other experimental sets. The histopathological result showed that embryos in the pre-treatment experimental set prevented pathological changes. This study indicated the ethanolic extract of RT can up-regulate the expression of Interferon-α genes and the anti-inflammatory cytokine IL-10 gene. Therefore, it prevents the spike protein of the delta SARS-CoV-2-induced pathological changes in fertilized chick embryos.

Keywords: SARS-CoV-2; Rhus toxicodendron; chick embryo; urushiol; interferons

Introduction

The Coronavirus disease 19 (COVID-19) is undeniably the biggest catastrophe the world has experienced in the 21st century. It was first reported in Wuhan, Hubei province of China, in late 2019 [1]. The health experts there reported cases of unusual pneumonia. Later, the causative agent was identified and named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). SARS-CoV-2 is a type of betacoronavirus with a single-stranded, non-segmented, positive sense RNA genome [2,3]. The virus enters the host cell by receptor-mediated endocytosis; when the receptor binding domain (RBD) of the virus binds with angiotensin-converting enzyme (ACE) 2, the procedure is triggered [4,5]. Following the entry of the viral material, the host's innate immune response is initiated through the expression of type 1 interferon (IFN) within the cell. In the first step, immune cells recognize the viral components (viral RNA) with pattern recognition receptors (PRRs), then toll-like receptor (TLR)- 7, 8, RIG-I recognizes the RNA virus. These produce anti-viral IFN (type I & III) and chemokines [6,7]. In COVID-19 patients, the fatality depends on the development of cytokine storms. The

hyperinflammatory state is developed by the rapid proliferation and hyperactivation of T-cells, natural killer (NK) cells, macrophages, and uncontrolled production of pro-inflammatory cytokines (IL-6, IL-8, IL-1β, TNF-α& GM-CSF) and chemokines (CCL-2, CCL-3, CCL-5, IP-10) causes damage in lung alveolar epithelium membrane, microvasculature that leads to ARDS and death. So, the main concern lies in combating this hyperinflammatory state to reduce the mortality of COVID-19 [8-15]. Traditional treatments were also used to manage mild to moderate COVID-19 cases. Medicinal plants have been integral to conventional medicine since the ancient era. Flavonoids, polyphenolic compounds, alkaloids, and essential oils of different medicinal plants exhibited promising results about COVID-19 [16-18].

Trials conducted with a small cohort during the pandemic suggested that plant-based ethanolic extracts of Rhus Toxicodendron (RT), Bryonia alba, Pulsatilla nigricans, Nux vomica, and Gelsemium sempervirens could produce favorable results in COVID-19 affected participants [19,20]. However, many controversies have come along due to a need for more explanation of the mechanism of action. The exact target areas still need to be explored and need proper support of scientific explanation. However, scientifically-backed data were awaited to explain if those extracts can balance the abnormal cytokine expression in COVID-19. Fertilized chick embryo (Gallus gallus domesticus) as an experimental model was proven feasible and ideal for studying the alteration of cytokine gene expression, as shown in different studies. Furthermore, the fertilized egg model is easy to procure and maintain and exhibits a good amount of viral replication, which is complex in animal models. It also bears a limited ethical and legal issue (In this experiment 14th, day fertilized chick eggs were procured; ethical issues are not required up to 18 days) [21,22]. Goswami et al. showed ethanolic extracts of Bryonia alba could upregulate IFN – α, IFN- ß, and TGF – ß gene expression in fertilized chick embryos [23]. Moreover, studies in identical models with unalike interventions showed notable results in chick embryos [24,25]. This is a novel work as no such study of ultra-diluted Rhus Toxicodendron extract has been studied so far against SARS-CoV-2 pathogenesis to explore its immune-modulatory activity in the fertilized chick embryo model.

Toxicodendron sp., previously known as Rhus Toxicodendron, belongs to the family Anacardiaceae. More than 80 genera and 600 - 750 species are under the Anacardiaceae family, typically disseminated in tropical Africa, Asia, and the Americas. Plants of Genus Toxicodendron, such as Toxicodendron radicans (poison-ivy) and Toxicodendron diversilobum (poison oak), are the most essential sources for the extracts of RT. Several pre-clinical studies have shown that RT extracts have immunomodulatory, anti-inflammatory, and anti-arthritic activities. RT extracts, both in crude and series of dilutions, accelerated the metabolic activities of PMN cells by increasing the phagocytic activity [26,27]. RT also helps in the expression of COX2 mRNA and decreases NO generations in mouse pro-osteoblastic cell lines [28]. Several studies showed the efficacy of RT in reducing Carrageenan-induced rat paw edema [29]. RT chiefly comprises an active immunogen named Urushiol. Urushiol came from the Japanese word for lacquer tree (urushi), a combination of organic compounds and oily resin mixed within [30]. Numerous lipophilic catechol derivatives are present in this organic mixture, pentadecyl-catechols and heptadecyl-catechols being more abundant. Pentadecyl-catechols and heptadecyl-catechols are organic compounds with 15-carbon side chains (pentadecyl) or 17-carbon side chains (heptadecyl) (Figure 1) of different degrees of saturation that are attached to a ringed carbon structure, catechol ($C_6H_4(OH)_2$). Urushiol is chiefly known for causing irritating delayed allergic contact dermatitis, manifested by redness, swelling, and oozing, with a persistent itching sensation [31,32].

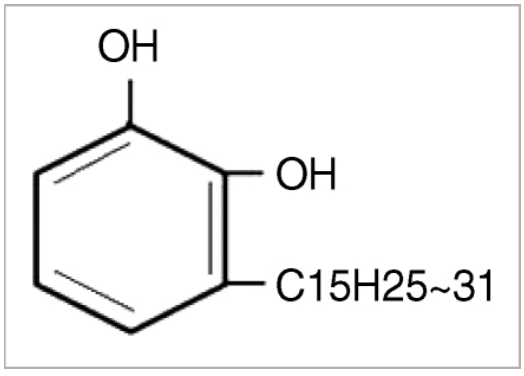


Figure 1. Chemical Structure of Urushiol.

Materials and Methods

The spike protein

For this study, the Delta SARS-CoV-2 spike RBD (L452R, E484Q) protein was manufactured by ABclonal Lot: 9621050601, Cat. No. RPO2266, Code: WH192258, was acquired. The HEK 293 expression system is used to produce this recombinant protein. The target protein is made up of the SARS-CoV-2 spike RBD sequence (Arg 319-Phe541) attached to a poly-histidine tag at the C-terminus (with mutations L452R, E484Q, Accession #YP-009724390.1).

Medicine & vehicle alcohol

For this study, both *Rhus Toxicodendron* (RT) (6CH potency according to homeopathic pharmacopoeia comprises material at attogram level) and vehicle alcohol were acquired from a government-accepted alternative medicine manufacturing company, "HAPCO, India".

Fertilized egg inoculation

14th day-old fertilized chick eggs (Gallus gallus domesticus) were obtained from the State Poultry Farm, Kolkata. At first, eggs were cleaned with distilled water. Next, air sacs were identified by candling and marked with a marker pen. The shells of those eggs were washed with 70% ethanol and 10% povidone-iodine solution. Then, using a sterile needle, small punctures were made at the center of the air sacs [33]. Every experimental set was then separated, a 100 µl volume of previously chosen materials was inserted via the amniotic route of the eggs, and identification marks were specified for each set (Given below). After the inoculation, the puncture sites were closed with a sterilized sticker. After that, all the inoculated eggs were incubated at 38 ºC at 60% humidity for 48 h. After 48 h, allantoic fluid (5 - 10 ml) was collected and stored at 80 ºC. Hepatic tissue was also dissected and preserved for further study.

The experimental sets were:

Set-1: 14th day embryonated eggs - **Control**

Set-2: Eggs challenged with 70% v/v molecular grade ethanol - **Alcohol control**

Set-3: Eggs challenged with original spike protein (S) RBD antigen - **Antigen control**

Set-4: Eggs challenged with RT 6CH - **Medicine control**

Set-5: RT 6CH was inoculated first, followed by antigen after one hour of incubation – **Pre-treatment Set**

Set-6: Antigen was inoculated first, followed by RT 6CH after one hour of incubation – **Post-treatment Set**

Estimation of cytokine expression

Comparative gene expression studies were determined after real-time PCR (Bio-Rad CFX96, Singapore) with SYBR Green tagged primers, dNTPs, Taq polymerase, MgCl2, buffer, etc. Chiefly, 8 cytokines genes, i.e., chicken Interferons (chIFN) α, β, γ; chicken Interleukins – chIL-1β, chIL-6, chIL-8, chIL-10; chicken Transforming Growth Factor (chTGF) β1 concerning β-actin were analyzed. Regarding RT PCR analysis, 2 µL of cDNA and 18µL of Taq universal sybr green supermix (Bio-Rad, USA) were mixed and analyzed, as per RT-PCR instrument following standard protocol [34].

Histological study

Paraffin blocks with the tissue samples were made after its fixation in formol saline, followed by using a microtome, 3 – 5 µm thick sections of it were made. After that, haematoxylin and eosin staining (H & E staining) was done following the standard guidelines [35].

Results

Changes in the Cytokine Gene Expression

In a concentration of 10 microgram/ml, delta SARS-CoV-2 spike protein RBD antigen increases the expression of the IFN- α gene by 1692-fold, but the expression of other cytokines was not significant in this study. In the other control set with Alcohol (70%), none of the cytokine gene expression was significant. In the experimental sets consisting *of Rhus Toxicodendron* 6CH (directly), pre-treatment, and post-treatment with *Rhus Toxicodendron*, the IFN- α and IL-10 expression were significantly increased. However, IL 6, IL 8 IL1B, IFN-beta, IFN-gamma, and TGF beta expressions were insignificant. Changes of all the cytokine gene expression is given in Table 1.

Table 1. Semi-quantitative increased gene expression of different cytokines in different experimental sets.

SETS	IFN- α	IFN- ß	IFN- γ	IL 8	IL 10	IL 1 B	TGF B1	IL 6
SET-1	0	0	0	0	0	0	0	0
SET-2	116.97	36.63	2.36	3.16	0	2.36	2.97	59.096
SET-3	1692.57	149.085	2.02	3.02	2.948	2.02	74.28	7.285
SET-4	17379.978	349.032	0.360	9.417	2244.961	0.513	0.000	52.235
SET-5	12245.677	27.074	523.236	26.172	5932.635	2.246	0.763	60.097
SET-6	14684.459	137.973	43.268	27.464	4117.113	1.179	4.832	58.311

General appearance of the chick embryo

Embryos of the Antigen control set were dead and putrefied. Pre-treatment and Post-treatment set embryos showed better vitality and growth than standard control. Lungs of all the embryos of each set were collapsed. Antigen control set embryos showed gross hemorrhagic areas at different parts when dissected. No significant macroscopic changes were found in all remaining sets. Different experimental sets with representative pictures of embryos are depicted in Figure 2.

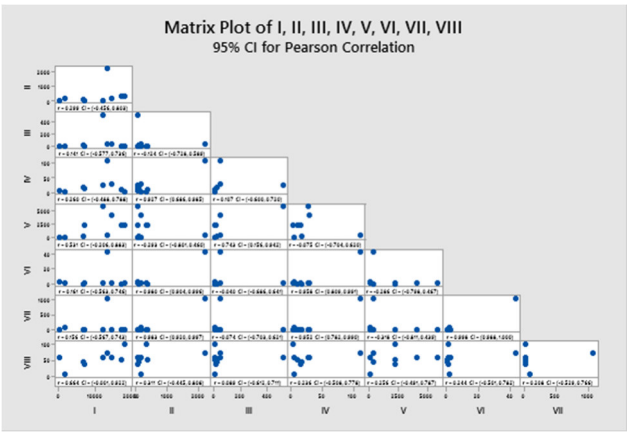
Figure 2. Macroscopic appearance of embryos in experimental sets. Set 1: control; set 2: alcohol control; set 3: medicine control; set 4: antigen control; set 5: pre-treatment set (Rhus Toxicodendron 6CH challenged by antigen); set 6: post-treatment set (antigen challenged by Rhus Toxicodendron 6CH).

Histopathological changes

With controlled alcohol, few necrotic changes are present. The antigen set has necrotic changes with many fibroblasts and microclots in the blood vessels. The liver is almost standard in the preventive (pre-treatment) set, with very few organized microclots in the blood vessels. In the curative (post-treatment) set, however, the necrotic changes and proliferation of sum fibroblasts are prominent.

Statistical analysis

Here, the numbers I – VIII indicates the following: I – Interferon alpha (IFN α), II – Interferon beta (IFN β), III – Interferon-gamma (IFN γ), IV – Interleukin 8 (IL -8), V – Interleukin 10 (IL -10), VI – Interleukin 1 beta (IL -1β), VII – Transforming growth factor beta 1 (TGF-β1), VIII – Interleukin 6 (IL -6) (Table 2 & 3).



Details of statistical analysis

Table 2. Correlations between group.

	I	II	III	IV	V	VI	VII
II	0.299						
III	0.141	-0.124					
IV	0.260	0.927	0.107				
V	0.531	-0.293	0.743	-0.075			
VI	0.161	0.980	-0.040	0.958	-0.286		
VII	0.156	0.983	-0.074	0.952	-0.318	0.998	
VIII	0.664	0.311	0.089	0.236	0.256	0.244	0.208

Table 3. Pairwise pearson correlations.

Sample 1	Sample 2	Correlation	95% CI for ρ	P-Value
II	I	0.299	(-0.456, 0.803)	0.435
III	I	0.141	(-0.577, 0.736)	0.718
IV	I	0.260	(-0.488, 0.788)	0.499
V	I	0.531	(-0.206, 0.883)	0.141
VI	I	0.161	(-0.563, 0.746)	0.679
VII	I	0.156	(-0.567, 0.743)	0.689
VIII	I	0.664	(-0.001, 0.922)	0.051
III	II	-0.124	(-0.728, 0.589)	0.751
IV	II	0.927	(0.686, 0.985)	0.000
V	II	-0.293	(-0.801, 0.460)	0.444
VI	II	0.980	(0.904, 0.996)	0.000
VII	II	0.983	(0.920, 0.997)	0.000
VIII	II	0.311	(-0.445, 0.808)	0.416
IV	III	0.107	(-0.600, 0.720)	0.784
V	III	0.743	(0.156, 0.942)	0.022
VI	III	-0.040	(-0.686, 0.641)	0.919
VII	III	-0.074	(-0.703, 0.621)	0.851
VIII	III	0.089	(-0.612, 0.711)	0.821
V	IV	-0.075	(-0.704, 0.620)	0.848
VI	IV	0.958	(0.809, 0.991)	0.000
VII	IV	0.952	(0.782, 0.990)	0.000
VIII	IV	0.236	(-0.508, 0.778)	0.541
VI	V	-0.286	(-0.798, 0.467)	0.456
VII	V	-0.318	(-0.811, 0.439)	0.404
VIII	V	0.256	(-0.491, 0.787)	0.505
VII	VI	0.998	(0.988, 1.000)	0.000
VIII	VI	0.244	(-0.501, 0.782)	0.526
VIII	VII	0.208	(-0.529, 0.766)	0.591

Discussion

Interferons (IFNs) are a group of prominent cytokines that have various biological responses. They contribute to innate and adaptive immunity to counter viral infections. IFNs are chiefly produced by lymphocytes, NK cells, B-cells, T-cells, epithelial cells, and hepatocytes. Likewise, SARS-CoV-2 IFNs trigger the immune response in other viral infections as the first line by recognizing the pathogen-associated molecular patterns (PAMPs). Data suggests IFNs play a crucial role in COVID-19 by preventing the progression to severe pneumonia [36,37]. It was seen that IFN alpha and IFN beta were highly reduced in severe COVID-19 patients. In this experiment, the expression of IFN alpha is significantly higher in the pre-treatment (>12200 folds increase), post-treatment (>14600 folds increase), and direct RT (>17300 folds increase) challenged sets (Table 1). But in the control group, consisting of the alcohol (70%), the change in expression was not significant enough. Thus, the effect of vehicle ethanol on the RT is ruled out. Antigen alone upregulated the IFN alpha gene by 1600 folds but was also insignificant in the RT groups. Therefore, the result shows RT has definitive curative and preventive properties in vitro. The chief anti-inflammatory cytokine, Interleukin-10 (IL-10), is a crucial cytokine for

the host immune response and prevention of tissue damage in the initial phase of the immune response. Again, IL-10 suppresses the pro-inflammatory cytokines in the recovery phase of viral infection in cases of immune system hyperactivation [38]. In the pre-treatment and post-treatment sets, IL-10 gene expression is upregulated by 5900 folds and 4100 folds, more than the RT (direct) set. There were insignificant responses to antigen and alcohol control sets. The presence of RT could upregulate the IL-10 gene expression. Yet, the expression of inflammatory cytokine genes (IL-6, IL-8, IL-1ß, TGF-ß) was not significant in all the experimental sets. In statistical analysis, there was also a significant correlation between IFN alpha and IL 10 cytokine (r = 0.531), adding an essential outcome to the results. Urushiol is the important bioactive component of RT; apart from its ability to make allergic contact dermatitis, it displays various biological activities that include anti-oxidative activity in non-alcoholic fatty liver disease [39], cytotoxic activity on human ovarian cells [40], antibacterial activity in different studies [41]. Urushiol components were able to down-regulate the expression of pro-inflammatory cytokine expression in Helicobacter pylori-induced infection in mice. Urushiol is also known to increase the urushiol-specific T-cells locally [42]. In severe COVID-19-affected patients, there is a decline in CD 3+ T-cells, and due to loss in CD 8 T cells, there is an inversion of the CD4 to CD8 ratio. So, T cells are potentially damaged by the virus. Here in this study, it can be hypothesized that RT can increase the urushiol-mediated T-cells and anti-inflammatory cytokines and maintain the cytokine milieu in the organism. However, this experiment cannot determine whether urushiol upregulates the T-cell receptor gene or inhibits the TLR4/MyD88/NF-κB signaling pathway during the hyperinflammatory state of COVID-19.

Conclusion

In this study, we elucidated that the diluted ethanol extract of Rhus toxicodendron6CH can up-regulate the expression of Interferon genes and the anti-inflammatory cytokine IL-10 gene. IFN alpha gene expression and IL 10 gene expression significantly increased in experimental sets directly treated with Rhus Toxicodendron 6CH, pre-treatment, and post-treatment sets. Therefore, this experiment also helps us to understand the curative and preventive effects of ethanolic extracts of Rhus Toxicodendron 6CH. Nevertheless, all phases of human trials are required before their acceptance for treatment in the population.

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Authors contribution

Author DC has carried out the experimental work. SG has written the manuscript, and PG, DC, and KP have also participated in the experimental process, especially during the collection of tissue and fluid samples and the harvesting of eggs. The author, SD, planned the entire experiment, analyzed the data findings, and revised the final manuscript. PA facilitated the conceptualization and execution of the whole work.

Declaration of interest

The authors declare no conflict of interest.

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