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Original Research Article

Ultra-Diluted Gelsemium Sempervirens a Known Dna Topoisomerase i (Top i) Inhibitor Exerts Protective Action Against Sars-Cov-2 Rbd Induced Cytokine Dysregulation

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Abstract: *Gelsemium sempervirens* (GS) extract is being used in phytomedicine and homeopathy for its anxiolytic properties but its mechanism of action is yet to be understood. Evidence from rodent models suggests existence of its high sensitivity to the central nervous system even in ultra-diluted conditions. The diverse effects of its extract and/or its main alkaloids-gelsemine, sempervirine, and koumine have been shown through different experiments in recent years. Sempervirine intercalates with DNA and inhibits topoisomerase-I activity, which is thought to be a potential target for restricting viral replication during SARS-CoV-2 pathogenesis. Delta SARS-CoV-2 spike RBD, the recombinant protein, was procured from Abclonal Pvt. Ltd. 14th-day-old *Gallus gallus domesticus* embryos were inoculated with RBD protein along with control alcohol in pre- and post-treatment sets and challenged with Gelsemium 6CH, 30CH and 200CH potencies. After 48h, allantoic fluids were collected during harvesting and stored at −20 °C for the study of different cytokine gene expressions by RT-PCR (Reverse Transcription Polymerase Chain Reaction). GS at 6CH, 30CH, and 200CH dilutions showed up-regulation of IFN-α and IL-10 gene expressions in all experimental sets. Tendencies of down-regulation of the genes were seen with TGF-β1, IL-1β, and IL-6 cytokines, with few exceptions. IFN-β and IL-1β gene expression changes were relatively mild and mostly inconclusive. All expressions indicate a possible balancing effect between pro-inflammatory and anti-inflammatory cytokine gene expressions by Gelsemium. Ultra-diluted GS in homeopathic doses can effectively modulate the expression of cytokine genes in SARS-CoV-2-induced cytokine imbalance. Further studies are desired to understand its utility in clinical practice through structured clinical trials.

Keywords: SARS-CoV-2; Spike Protein; Receptor Binding Domain (RBD); Cytokine imbalance; Gelsemium; Homeopathy

1. Introduction

The emergence of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has forced healthcare workers and medical researchers to recognize the critical role of an effective host immune response and the devastating effects of immune dysregulation. The lack of targeted therapy, along with the outbreak of mutant strains of coronavirus, made the situation even more severe. In response, different branches of medicine came

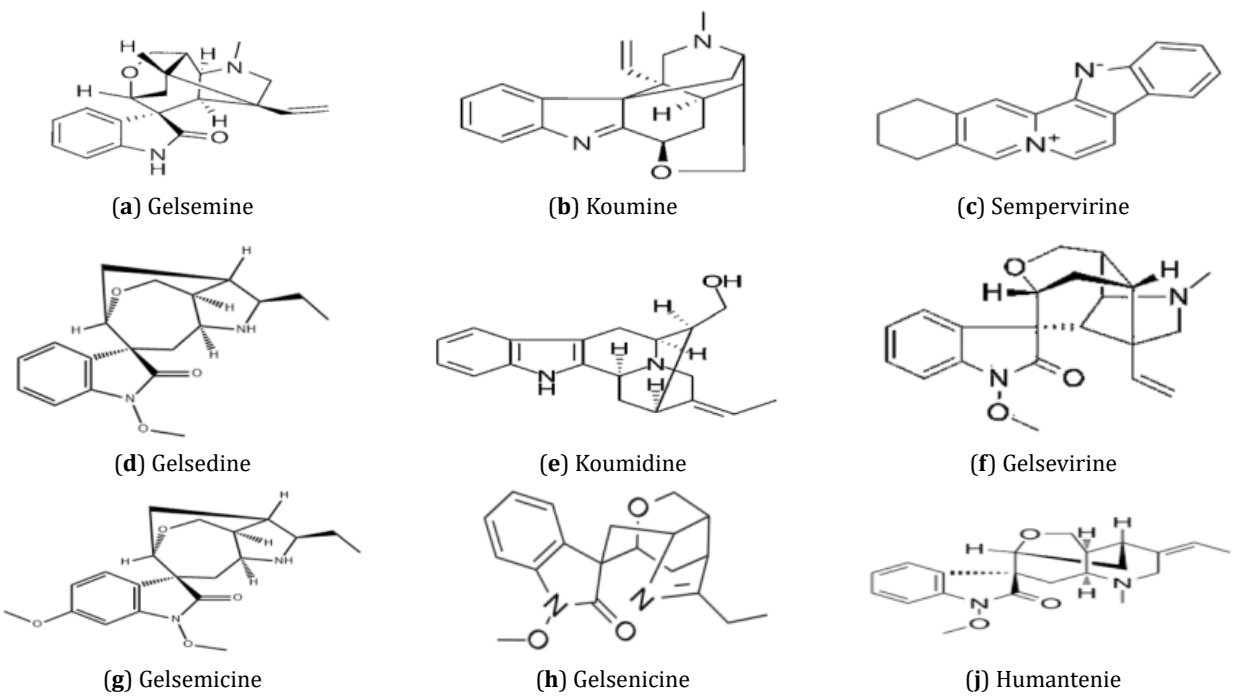
together to contribute in their unique way and homeopathy is one of them. Despite being criticized for its heterogeneous theories and practices that lack scientific validation, homeopathy has always shown a significant impact on users as per studies [1]. The colonial past of nations such as India and other Asian countries (China, Japan, Indonesia, etc.), which have long-standing traditional and complementary health systems, is one of the reasons for biomedicine's current supremacy in those systems. Based on a pluralistic approach to health care, India offers a spectrum of medical treatments, and homeopathy is an integral part of this framework [2]. The situation has gained more attention as a result of the active intervention of the World Health Organization (WHO) in changing its policy regarding traditional and complementary medicine, aligning it with evidence-based medicine (EBM) in the upcoming years [3]. During the pandemic, various medicines were speculated for coronavirus disease 2019 (COVID-19) in published homeopathic literature. However, prognostic factor research conducted by To et al. and Manchanda et al. identified *Arsenic album, Bryonia alba, and Gelsemium sempervirens* (GS) as medicines with significant outcomes and a greater likelihood ratio for the reported symptoms of COVID-19 [4, 5]. All these publication falls short when it comes to judicial explorations about mechanisms of action of these homeopathic medicines. Despite the fact that medical science has evolved since the inception of EBM in the early 20th century, unfortunately, that very intent of studying different branches of drug pathogenesis in depth is not much evident in homeopathic research.

GS is a flowering plant in North America that belongs to the family Gelsemiaceae. Though GS is commonly referred to as a toxic plant, its long use in traditional medicine today speaks in favor of its undeniable beneficial effects. The documented therapeutic application of GS dates back to the nineteenth century, when the plant was wrongly identified as an alternate herb for treating a man with "bilious fever" [6]. Homeopathic use of GS mostly depends on its proven symptoms in the sphere of the nervous system (anxiety, neuralgia, migraine), renal system (to increase output), infective diseases, and respiratory illnesses (asthma, whooping cough, etc.) [7]. Evidence from both animal and cellular research has revealed that the central nervous system was highly sensitive to the effects of this plant, which were found even at extremely low dosages and high homeopathic dilutions (e.g., 5CH and 9CH) [8]. At present, 121 different alkaloids have been extracted from the genus, which has potential anti-tumor, analgesic, anxiolytic, anti-inflammatory, and immune-modulating activities [9]. During the phase of COVID-19, scientists had to look for numerous therapeutic strategies (anti-viral drugs, Interleukin (IL)-6 blockers, monoclonal antibodies, HOX-1 modulators, antibody cocktails, etc.) to combat SARS-CoV-2. DNA Topoisomerase I (Top I) was the one being thought to have a potential role in the viral replication and pathogenesis of SARS-CoV-2 [10]. Studies have also shown that it can inhibit the activity of human DNA Top I, which we think is the reason for its beneficial therapeutic outcome clinically [11, 12]. As researchers, we believe that EBM is a lifelong, self-directed, problem-based learning process in which caring for patients necessitates the acquisition of clinically important information about diagnosis, prognosis, therapy, and other clinical and health care issues [13]. To fill the existing caveat in the ongoing research, we must develop a clear understanding regarding the scope and limitations of these ultra-diluted medicinal compounds without being preconceived to overlook their efficacy as the 'Placebo effect'. Previously, we reported the cytokine modulatory effects of *Arsenic album* and *Bryonia alba*; here we are reporting the effects of GS against the pathogenesis caused by the SARS-CoV-2 spike protein receptor binding domain (RBD) [14–16]. In this experiment, we have attempted to understand the anti-viral effect of homeopathic medicine GS in different potencies (i.e., 6CH, 30 CH, and 200CH) against SARS-CoV-2 spike protein RBD-induced pathogenesis in *Gallus gallus domesticus* embryos.

2. Phytochemicals of Gelsemium

GS is an early-flowering twining vine that grows in the highlands of southern Mexico, Guatemala, and the coastal regions of the eastern United States [17]. It goes by several names, including yellow jessamine, Carolina jasmine or jessamine, and evening trumpet flower. Phytochemical studies on GS extracts have revealed at least 120 alkaloids, with nuclei consisting of bisindole, oxindole, or indole [9, 18]. Gelsemine, koumine, gelsemicine, gelsenicine, gelsedine, sempervirine, koumidine, koumicine, and humantenine (**Figure 1**) are among the most common indole alkaloids [9]. **Figure 1** includes the major alkaloids of gelsemium along with their chemical structure. These six types of alkaloids—gelsemine-type (gelsemine, gelsevirine), koumine-type (koumine), gelsedine-type (gelsedine), humantenine-type (humantenine), yohimbane-type (sempervirine), and sarpagine-type (koumidine)—are categorized based on their chemical structures [9, 19]. Researchers and pharmacologists have shown a lot of interest in the indole alkaloids that were isolated from GS owing to their intricate structural characteristics and va-

riety of biological effects. The main active ingredients in gelsemium are indole alkaloids, which include gelsemine, koumine, gelsemicine, gelsenicine, gelsedine, sempervirine, koumidine, koumicine, and humantenine. Numerous investigations have demonstrated the diverse biological properties of GS extracts and active alkaloids, encompassing immunosuppressive and anticancer actions [20–25]. In addition, hysteria, dysmenorrhea, chorea, whooping cough, asthma, skin ulcers, pneumonia, and bronchitis have all been treated with GS [26]. Recent research by Magnani et al. has shown that mice treated with a series of centesimal dilutions of GS prepared in accordance with homeopathic pharmacopeia exhibit anxiolytic-like effects [24]. The said medicine has been shown in in vitro studies to alter mice's emotional reactions to unfamiliar surroundings, which promotes exploratory behavior and reduces thigmotaxis or neophobia [24, 27]. The efficacy of ethanolic extracts of GS to control various types of neuropathic pain via blocking the glycinergic receptors is also well established [9]. Despite all this evidence, what has drawn our attention is the ability of the alkaloids in GS to inhibit the activity of human DNA Topoisomerase-I (Topo-I). Previously, it was demonstrated that sempervirine has the ability to intercalate with human DNA, which has been reproduced in another experiment carried out by Zhang et al. and Caprasse et al. in recent times that shows the intercalation of sempervirine and ethidium bromide (a well-known DNA intercalator) at different concentrations on relaxed DNA [11, 28]. Having said that, we also believe all this evidence fails to show the effect of GS on the acute phase reactants and is yet to be explored for its judicial application in acute illnesses. Cytokines (ILs, interferons, etc.) are the acute-phase reactants that remain elevated in respiratory infections such as influenza, SARS-CoV-2 infection, or late-stage malignancies [29]. In this experiment, we intended to carry out a novel endeavor to understand the impact of homeopathic preparations of GS on controlling cytokine gene expression in spike protein RBD-induced pathogenesis.



(**a**) Gelsemine (**b**) Koumine (**c**) Sempervirine

(**d**) Gelsedine (**e**) Koumidine (**f**) Gelsevirine

(**g**) Gelsemicine (**h**) Gelsenicine (**j**) Humantenie

Figure 1. The chemical structures of different alkaloids of *Gelsemium* sp.

3. Materials and Methods

3.1. The Recombinant Antigen

For this experiment, ABclonal Lot: 9621050601, Cat. No. RPO2266, Code: WH192258, produced the Delta SARS-CoV-2 spike RBD (L452R, E484Q) protein, which was directly procured. This recombinant protein is synthesized using the HEK 293 expression system. The SARS-CoV-2 variant (also known as B.1.617), which first appeared during the second wave in India, carried the alterations that were discovered. The protein was lyophilized and

filtered to a 0.22μm solution in Phosphate Buffered saline (PBS) at a pH of 7.

3.2. The Embryonated Eggs

Three-day-old embryonated *Gallus gallus domesticus* eggs were procured from the Government State Poultry Farm, Kolkata, India. Every egg went through precautionary measures to ensure it was pathogen-free. Before being candled, the eggs were gently cleaned with distilled water and then rectified spirits. Following that, it was placed in the incubator where temperature was lowered to 38 °C and the humidity was kept between 60 and 80%. To track the growth of the embryo in a dark environment, the eggs were candled twice a day. Eggs were split into six groups on the fourteenth day: antigen control, vehicle control (i.e., 90% alcohol volume/volume), GS 6CH, 30 CH, and 200 CH therapeutic and prophylactic groups. In the prophylactic group, the medicine was administered first, then, after one hour, antigen was administered. In the therapeutic group, the antigen was administered first, then, after one hour, the medicine was administered. Except for the usual control group, all other groups received 100 μL of antigen and drug substance via the amniotic route. The eggs were candled the next day after inoculation, rotated three times during the day, and incubated as described previously. Harvesting of all the eggs was done after 48 h (on the 16th day) following exposure at 2–8 °C for 2 h. For further investigation, 5–10 mL of allantoic fluids was collected in sterile vials and refrigerated at –80 °C. All experiments and assays were done in triplicates, and mean values were considered for analysis.

3.3. Medicines and Vehicle Alcohol

GS 6CH, 30 CH and 200 CH were directly purchased from Hahnemann Publishing Company Pvt. Ltd. (HAPCO), a GMP-certified company that follows the Homeopathic Pharmacopoeia of India for preparation of medicines.

3.4. RNA Extraction of the Samples

For the purpose of increasing the allantoic fluid's temperature, the samples were warmed in the water bath. To prevent any form of Ribonucleic Acid (RNA) contamination, the laminar airflow and associated equipment were cleaned with RNA-ase ZAP prior to RNA extraction. A centrifuge tube containing 200 μl of the allantoic fluid was filled with 1 ml Trizol and kept at room temperature for five minutes. The mixtures were then well mixed by vortexing. The tube was centrifuged at 12,000 rpm for five minutes at 4 °C after first being incubated for five minutes at room temperature. The supernatant was taken out once centrifugation was finished. Another 200 μl of chloroform ($CHCl_3$) was added, and the mixture was centrifuged for 15 minutes at 4 °C and 12,000 rpm in order to further separate the protein, RNA, and DNA. Three separate layers of protein, DNA, and RNA were seen following centrifugation. The translucent RNA layer was taken out and put in another sterile centrifuge tube using a sterile micropipette. Using a vortex, the separated fluid for RNA purification was thoroughly mixed with 500 μl of isopropanol (C_3H_8O). After ten minutes at room temperature, it was centrifuged a second time at 4 °C for ten minutes at a speed of 12,000 rpm. Following the removal of the supernatant, 750 μl of cooled ethanol was used to rinse the RNA before it underwent another vortex. The sample underwent one final centrifugation at 7500 rpm for five minutes at 4 °C. The supernatant was disposed of once again. To dry the collected RNA, the pellet was stored at 4 °C for the entire night.

3.5. RNA Estimation Procedure

The extracted RNA was stored at 4 °C overnight and the next day 60 microlitres of nuclease free water was added. The samples were incubated at 56 °C for ten minutes to dissolve the RNA. Thereafter, RNA purity was examined with the A260/280 ratio. If the ratio came within 1.8–2, the extracted RNA was considered to be pure. Then, each PCR tube is filled with 4 μl of the reverse transcriptase master mix, which is added and aspirated with the help of a pipette to mix the RNA with the nuclease-free water following the standard protocol of the manufacturer. The PCR tubes were then put into a thermal cycler (T100 BIORAD, USA) to produce complementary c-DNA, following the kit PCR-based program and the newly made c-DNAs were stored at – 20 °C for later use.

3.6. DNA Sample Preparation for Extracted RNA

We estimated the amount of water and RNA that must be mixed to produce 1 μg/ml of complementary DNA (c-DNA), using the OD260/OD280 ratio. Each PCR tube is filled with 4 μl of the reverse transcriptase master mix,

which is added and stirred together to homogenize the RNA and water in accordance with the standard protocol. The PCR tubes were then put into a thermal cycler (T100 BIORAD, USA) to produce complementary c-DNA, and the newly made c-DNAs were stored for later use.

3.7. Quantification of Gene Estimation by RT-PCR

The cDNA synthesized were used to perform gene expression analysis of the following cytokines namely interferons (IFNs) (α, β, γ) and ILs (IL-10, IL-6, Il-1β) were assessed through Reverse-transcriptase Polymerase Chain Reaction (RT-PCR) (**Table 1**). The assay was done using iTaq SYBR green kit (BIO-RAD, USA) following the detailed manufacturer's protocol. 2 microlitres of specific primers were mixed with 18 microlitres of PCR master mix, and the RT-PCR (CFX-96 model, BIO-RAD, USA) was conducted.

Table 1. Forward and reverse primer sequences used for the measurement of expressions of cytokine genes in RT-PCR in this experiment.

Cytokine	Forward Primer	Backward Primer
IFN-α	ATGCCACCTTCTCTCACGAC	AGGCGCTGTAATCGTTGTCT
IFN-β	CCTCCAACACCTCTTCAACATG	TGGCGTGCGGTCAAT
IFN-γ	CAAGTCAAAGCCGCACATC	CGCTGGATTCTCAAGTCGTT
IL-6	GCGAGAACAGCATGGAGATG	GTAGGTCTGAAAGGCGAACAG
IL-8	GCCCTCCTCCTGGTTTCAG	TGGCACCGCAGCTCATT
Il-1β	GCTCTACATGTCGTGTGTGATGAG	TGTCGATGTCCCGCATGA
IL-10	CGGGAGCTGAGGGTGAA	CGCTGGATTCTCAAGTCGTT
TGF-β1	TGCCACTCGCAAACATCTACG	GCAACTCAAACAGGGTCTTAGC

3.8. Estimation of Expressions of the Cytokines

Real-time PCR (Bio-Rad CFX96, Singapore) was used to evaluate comparative gene expression with SYBR Green-tagged primers, dNTPs, Taq polymerase, MgCl2, buffer, and other ingredients. Expression changes were assessed as a fold increase or reduction from the normal control and compared to the housekeeping gene, β-actin.

3.9. Ethics Approval

Although there are no ethical concerns with experiments conducted on embryonated eggs up to 18 days, we initially obtained clearance from the Institutional Ethical Committee, and the committee confirmed that no ethical issues arise if experiments are done within 18 days. In this experiment, we harvested embryonated eggs on the 16th day when they are not recognized as animals by animal welfare legislation. Thus, the question of potential harm or benefit to the animal does not arise as the embryo was sacrificed by the 16th day following all ethical parameters (by freezing). After completion of the process of harvesting and sample collection, the embryo and the other dissected body parts were disposed of by maintaining the bio-safety guidelines of our Institute which follows IACUC (International Animal Care and Use Committee) guidelines. The embryonated egg model in this condition may reduce or replace the use of small animals and currently enables the refinement of many experimental protocols.

3.10. Statistical Analysis

The statistical test was performed to assess the correlation between different groups and determine significant changes.

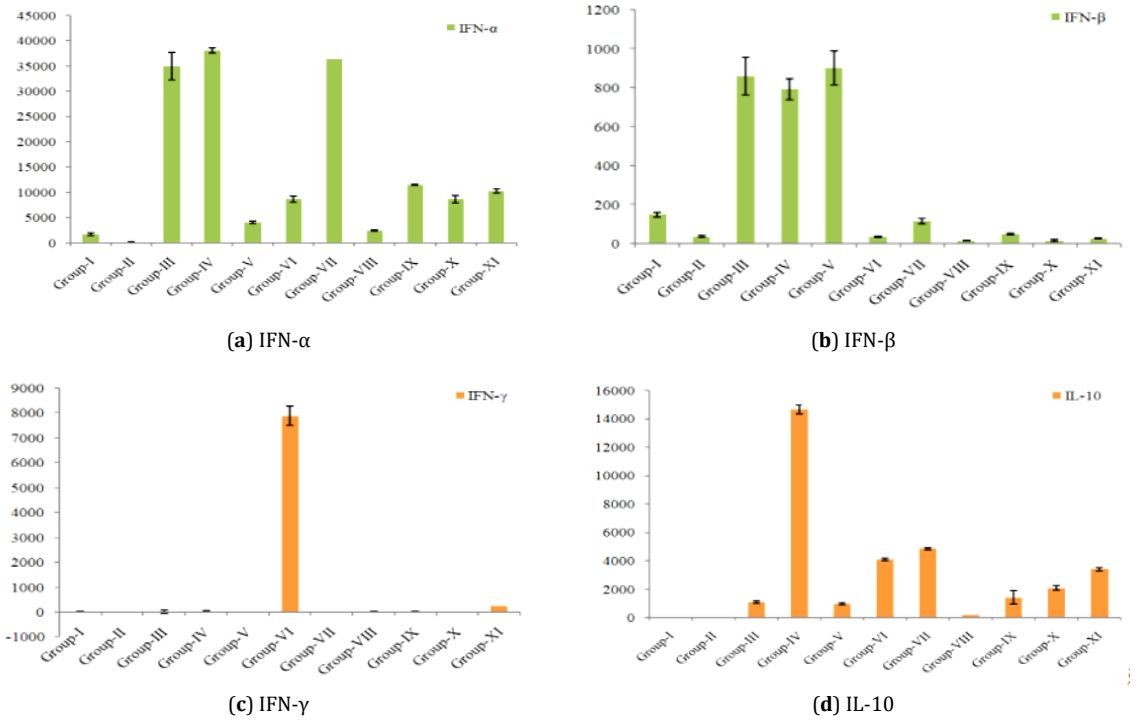
3.11. Results

Mild up-regulation in the expression of cytokine genes was seen with administration of GS 6CH, antigen (AG), or control alcohol (ALC). The administration of GS 6CH brought about a significant rise in the gene expression of TGF-β1, IL-10, and IFN-α in both prophylactic and therapeutic groups. On the other hand, IFN-γ and IFN-β were shown somewhat up-regulated along with GS 6 CH-induced down-regulation of IL-6 gene expression. Additionally, GS 30CH caused upsurge in both the preventive and therapeutic groups which had increased levels of IL-10 and INF-α. Nonetheless, we noticed differences in the expression of IL-6 and IFN-γ between the GS 30 CH prophylactic

and therapeutic groups. Expression of IFN-γ was markedly increased in the therapeutic group of GS 30 CH with down-regulation in the expression of IL-6, whereas the opposite was seen when GS 30 CH was administered as a therapeutic agent. Changes in the expression of cytokine genes with GS 200 CH were observed to be insignificant as IFN-α and IL-10 were up-regulated but the expressions of the rest of the cytokines were insignificant to claim their efficacy. Changes in the gene expression of IL-8 and IL-1β were found to be insignificant in all the sets. The result of our study suggests GS 6CH can be considered therapeutic and GS 30CH can be considered a potential therapeutic agent to combat SARS-CoV-2-induced pathogenesis. **Table 2** contains the fold expression (Mean ± SD) of cytokine genes and **Figure 2** shows graphical representation of the expression of cytokines in different sets of this experiment.

Table 2. Fold expression of cytokine genes in different sets of this experiment.

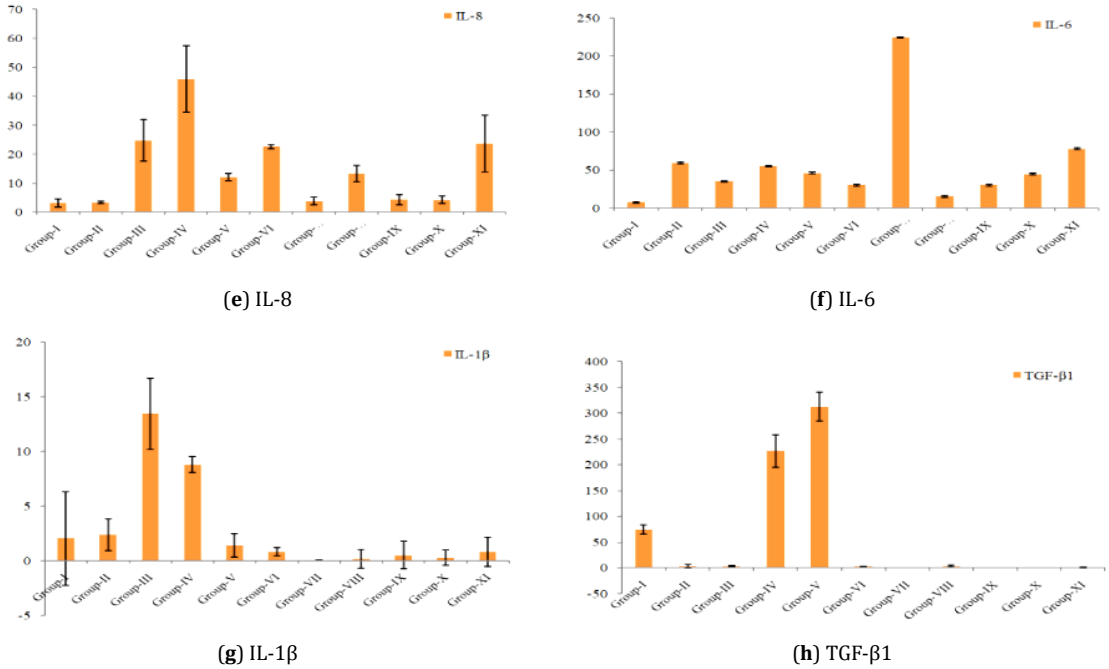
SETS	IFN-a	IFN-β	IFN-y	IL-8	IL-10	IL-1β	TGF-β1	IL-6
Group-I	**1692.57 ± 274.37**	149.09 ± 12.97	2.02 ± 1.37	3.02 ± 1.37	2.95 ± 1.63	2.02 ± 4.27	74.28 ± 9.34	7.29 ± 0.12
Group-II	116.97 ± 37.43	36.63 ± 4.61	2.36 ± 2.35	3.16 ± 0.39	3.28 ± 0.23	2.36 ± 1.47	2.97 ± 3.23	59.1 ± 3.15
Group-III	34948.84 ± 2730.27	861.74 ± 97.64	12.09 ± 76.25	24.64 ± 7.25	1084.95 ± 87.61	13.41 ± 3.27	3.2 ± 1.03	34.9 ± 4.65
Group-IV	38039.09 ± 473.83	793.04 ± 53.48	39.07 ± 2.73	45.87 ± 11.47	14653.46 ± 323.76	8.76 ± 0.73	226.28 ± 31.76	54.9 ± 7.32
Group-V	3996.42 ± 273.17	902.36 ± 87.63	7.89 ± 0.37	12.07 ± 1.23	969.02 ± 77.43	1.38 ± 1.07	311.91 ± 27.91	45.9 ± 2.79
Group-VI	8622.44 ± 643.25	35.83 ± 4.67	7888.80 ± 376.26	22.50 ± 0.73	4082.04 ± 89.91	0.79 ± 0.38	2.50 ± 0.37	29.90 ± 3.87
Group-VII	38928.78 ± 973.41	116.04 ± 13.73	32.27 ± 2.31	3.73 ± 1.23	4837.81 ± 67.43	0.01 ± 0.03	1.27 ± 0.31	224.12 ± 47.23
Group-VII	2443.95 ± 127.77	16.05 ± 1.478	13.28 ± 0.97	13.17 ± 2.84	191.34 ± 11.38	0.16 ± 0.87	3.13 ± 1.37	15.13 ± 1.29
Group-IX	11465.41 ± 149.97	49.69 ± 3.73	14.42 ± 3.28	4.2 ± 1.76	1398.83 ± 470.65	0.48 ± 1.27	0.23 ± 0.07	29.86 ± 2.49
Group-X	8629.13 ± 763.41	16.51 ± 9.73	1.32 ± 1.09	4.14 ± 1.37	2091.03 ± 157.25	0.27 ± 0.71	0.3 ± 0.18	44.32 ± 3.73
Group-XI	10261.83 ± 479.39	25.55 ± 7.83	243.88 ± 2.73	23.59 ± 9.73	3396.89 ± 117.73	0.79 ± 1.34	0.51 ± 1.38	77.71 ± 11.57



(**a**) IFN-α (**b**) IFN-β

(**c**) IFN-γ (**d**) IL-10

Figure 2. *Cont.*



(**e**) IL-8 (**f**) IL-6

(**g**) IL-1β (**h**) TGF-β1

Figure 2. A graphic representation of the fold expression of different cytokine genes across various experiment sets.

While IFN-β (**Figure 2b**) was mostly expressed with GS 6cH, IFN-α (**Figure 2a**) has been shown to be increased with GS 6cH and 30cH. Not on any other settings of this study, but with a post-treatment group of GS 30cH, IFN-γ (**Figure 2c**) was strongly expressed. With GS 6cH and a post-treatment group of GS 30cH and 200cH, IL-6 (**Figure 2d**) was down-regulated. Gels 30cH was found to down-regulate TGF-β1, whereas GS 6cH was found to up-regulate it (**Figure 2e**). With the injection of GS 6cH, IL-10 (**Figure 2g**) was elevated, while the expression was barely noticeable in the other groups. Comparing the expression of Il-1β (**Figure 2f**) and IL-8 (**Figure 2h**) to that of other cytokines, they are both uneven and negligible.

Statistical analysis: The Shapiro-Wilk test confirmed that cytokine data do not follow a normal distribution ($p < 0.05$ for all variables). Hence, Spearman's correlation (**Figure 3**) was the best choice because it accommodates non-normality, non-linearity, and small sample sizes, making it more robust for cytokine interaction analysis in this dataset.

The Spearman correlation analysis reveals strong positive relationships between IFN-α and IL-10 (0.818), suggesting a linked regulatory mechanism, as well as between IL-1β and TGF-β1 (0.747), indicating their close association in immune responses. IFN-β also moderately correlates with TGF-β1 (0.673), implying a functional connection in inflammation. Moderate correlations exist between IFN-α and IFN-γ (0.427), IFN-α and IL-6 (0.409), IFN-γ and IL-8 (0.618), and IFN-γ and IL-10 (0.564), reflecting potential interactions in cytokine signaling. Meanwhile, weak or negligible correlations, such as IL-8 and IL-6 (−0.009) or IL-1β and IL-6 (−0.009), suggest independent activity. Weak negative correlations, including IFN-α and TGF-β1 (−0.264), IL-10 and IL-1β (−0.214), and IL-10 and TGF-β1 (−0.236), hint at possible opposing immune functions. Overall, IFN-α and IL-10 show the strongest positive correlation, while TGF-β1 appears to have significant links to both IFN-β and IL-1β, with some cytokines such as IL-8 and IL-6 operating independently.

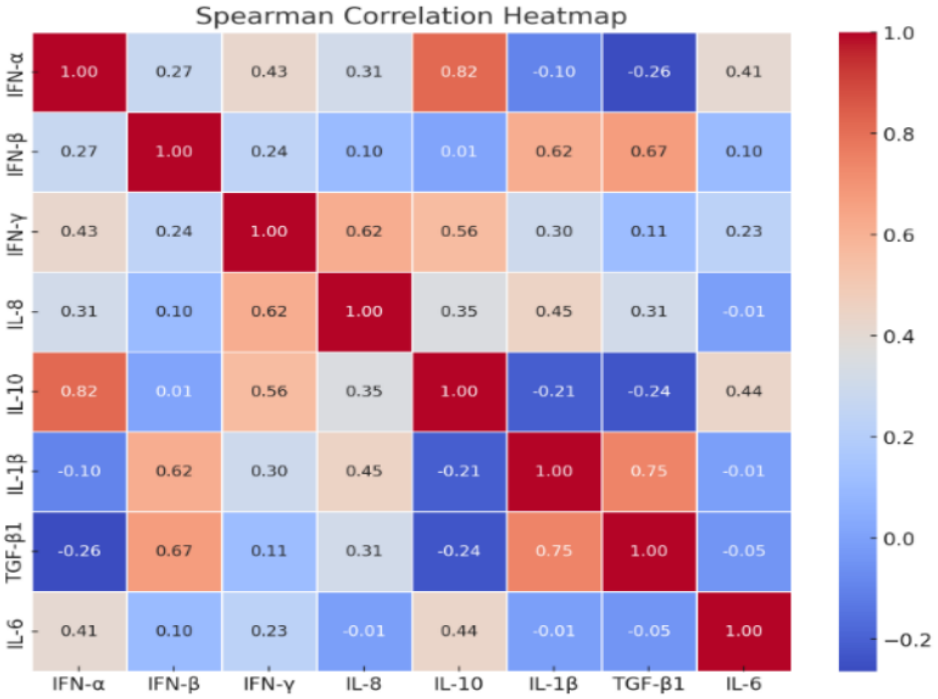


Figure 3. The Spearman correlation heatmap showing strength and direction of monotonic relationships between variables.

4. Discussion

The patho-physiological spectrum of cytokine storm can be referred to as a state of altered immune response that encompasses general and systemic symptoms which is responsible for poor clinical outcome in COVID-19. The onset and duration of cytokine storm is indeed variable depending on the time of administration of intervention but its undeniable influence in the pathogenesis of COVID-19 has been contemplated in different studies [30, 31]. The primary factor influencing the fate of SARS-CoV-2 infection is its attachment to the cell surface and subsequent entry into the cell, which is further influenced by the interaction between the ACE-2 receptor and two subunits (S1 and S2) of the SARS-CoV-2 Spike protein [32–35]. Studies have shown that SARS-CoV-2 spike protein interacts with the ACE-2 receptors, and previous cryo-electron microscope studies of this interaction have demonstrated that RBD causes the S1 to break away from ACE-2, causing the S2 to transition from an unstable, pre-fusion state to a more stable post-fusion state, which is necessary for membrane fusion [36–39]. SAR-CoV-2 RBD exhibits a greater affinity for binding with ACE-2 receptor in a low nano-molar range which is the reason for RBD is considered a crucial functional element of the S1 subunit that is in the charge of binding SARS-CoV-2 with ACE-2 [40, 41]. Following entry, SARS-CoV-2 primarily infects airway and alveolar epithelial cells, particularly type II pneumocytes, as well as cells that express the ACE-2 receptor, including endothelial cells, pericytes, vascular smooth muscle cells, macrophages, fibroblasts, T-cells, cardiomyocytes, enterocytes, basal cell epidermal cells, and epithelial tubular distal cells [42–44].

During the early stage of infection, SARS-CoV-2 can replicate in high titers when its replication is unregulated due to impaired innate immunity [45, 46]. The excessive accumulation of neutrophils and inflammatory monocyte-macrophages in the lungs after infection with SARS-CoV-2 encourages the release of more chemokines and cytokines. From localized inflammation, cytokines spread throughout the entire body. COVID-19 patients have elevated levels of inflammatory cytokines such as IL-2, IL-6, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), IP-10, monocyte chemo-attractant protein (MCP-1), macrophage inflammatory protein (MIP-1A), and Tumor Necrosis Factor-α (TNF-α), which are not only of diagnostic but also correlate with severity hence holds prognostic value [47, 48]. The destruction of the alveolar bed and changes in permeability that result in vascular leakage and alveolar edema are mostly caused by this increase in cytokines. To combat the situation different

therapeutic strategies (Anti-viral drugs, IL-6 blockers, monoclonal antibodies, Antibody cocktail, plasma transfusion, etc.) were adopted but nothing seemed to be sufficient. However, evidence suggests that inhibition of single cytokines such as IL-6 or GM-CSF might not be sufficient [49, 50]. This is because many signaling molecules and pathways are involved in triggering an inflammatory response. Additionally, levels of individual cytokines can vary depending on the age and the clinical history of the patient, thus limiting the scope of therapeutics that only target a single inflammatory molecule. It is better to look for an alternative that can restrict the viral replication which in turn can arrest the cascade of immune dysregulation and its later consequences. For the same, Top-I inhibitors were considered and tested among the different in-vitro and in-vivo models with significant outcomes in favor of their application [51]. Unfortunately, despite being mentioned in several studies, to date, no published clinical trial is available with Top I inhibitors in COVID-19.

Previously, it has been seen in multiple studies that the phytochemicals of *GS* can act as DNA Top-I inhibitors [52]. Top-I has also been argued to play a pivotal role in the innate immune response as Top-I reportedly suppressed the pro-inflammatory immune response against pathogenic infections at cellular and organismal levels. Rinaldi et al. performed a chemical screen for innate immune system-intrinsic regulators of the transcriptional response to pathogens and observed the inhibitory activity of Top-I inhibitor, camptothecin, on the expression of pro-inflammatory genes [51]. Significantly, the inflammatory immune response against many bacterial and viral pathogens and their collateral damage was compromised by suppression of Top-I activity. A comprehensive investigation by Ho et al. revealed the necessity of assessing topotecan, a Top-I inhibitor, for treating severe COVID-19 in humans. Treatment with two doses of topotecan reduced the fatal inflammation caused by SARS-CoV-2 in animal models [10]. Besides that, studies have reported that nsp2 of SARS-CoV-2 has a molecular mimicry and functional similarity with DNA Top-I [52]. Chakraborty reported a short homology of Nsp2 protein with DNA primase, gyrase A and gyrase B, DNA gyrase subunits, indicating that the Nsp2 protein has acquired RNA/DNA binding, nickase and ligase domains from different regions of the related genes during its creation [52]. That also stands out as a substantial reason for considering application of Top-I inhibitors in COVID-19. An "infection-induced gene program"—is a planned elevation of anti-viral and inflammatory mediator expression that is triggered by every infection. The degree of gene expression can influence the innate immune response and viral antagonism in the cell, which can have paradoxical effects on the host cells. Balanced expression of these pro and anti-inflammatory cytokines during the process of inflammation is an important mediator for the effective therapeutic outcome. IL-10 is considered as master moderator of anti-inflammatory response. In this experiment marked up-regulation in the expression of IL-10 and IFN-α along with subsequent down-regulation in IL-6, TGF-β1, IL-8 and IL-1β in the therapeutic group with GS 6CH may be due to the Top-I suppression. However, the down-regulation of IFN-γ raises concern for the application of Gelsemium 6CH as prophylactic in COVID-19. On the other hand, marked upsurge of IFN-γ, along with moderate up-regulation in IFN-α and subsequent down-regulation of IL-6, TGF-β1, IL-8 and IL-1β, stands in favor of the application of Gelsemium 30 CH as prophylactic for COVID-19.

The current trend in research on GS is mostly directed at evaluating its nociceptive property, anxiolytic effects and its ability to alleviate neuropathic pain. Though homeopathic preparations of Gelsemium have been used in COVID-19, to date, no studies have been conducted to understand its behavior to control the pathogenesis or virus-induced hypercytokinemia. This experiment is a novel endeavor to explore the therapeutic utility of ultra-diluted GS in different concentrations against SARS-CoV-2 spike protein RBD-induced pathogenesis.

5. Conclusion

Rapid increase of gene expression is a key factor in triggering an inflammatory response. Inhibition of this mechanism may hold the key to developing innovative therapies for COVID-19. We truly believe that there is no alternative in medicine; if a substance is efficacious then it should be explored in every aspect to understand the scope and limitations of its use. We believe our study will help to understand the applicability of different potencies of GS in COVID-19. The dose-dependent variation in action is possibly reported for the first time as it needs further study before administering these ultra-diluted medicines empirically. The need for a structured clinical trial is also undeniable for judicious application of this medicine on a large scale to combat future outbreaks.

The main caveat for this research is that it was done on the *Gallus gallus domesticus* embryo. The immune response of the *Gallus gallus* embryo is somewhat similar to that of humans but it is not an exact representation. Moreover, we went on with the viral antigen, but the inoculation with the live virus could have given a better repre-

sentation. To obtain a better knowledge of cytokine gene expression, models such as human organoids and in-vivo experiments could be taken into consideration.

Author's Contribution

Experimental work: D.C.; manuscript writing: P.G.; experimental process (inoculation, collection of tissue and fluid samples during egg harvesting): P.G., S.G., K.P.; experiment planning, data analysis, manuscript revision: S.D. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The data and materials have been made available.

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Conflict of interest

The authors declare no conflict of interest.

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