

*Full Length Research Paper*

# Preclinical immunomodulatory activity of COVIDEX<sup>®</sup> herbal product developed for supportive treatment of COVID-19 in Uganda

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Received 23 September, 2023; Accepted 19 June, 2024

The outbreak of the COVID-19 pandemic due to the SARSCoV-2 virus caused international lockdown with devastating effects on global health. In Africa, most people resorted to the use of herbal products for the prevention and treatment COVID-19 related symptoms. In Uganda COVIDEX<sup>®</sup> herbal product was widely used and is still used against COVID-19 and other viral symptoms of the upper respiratory tract. The product contains extracts of plants with berberine an anti-SARSCoV-2 compound as one of the isoquinoline alkaloids in it. The product is formulated using extracts of *Zanthoxylum gilletti*, *Warburgia ugandensis*, and *Cymbopogon citratus* plants indicated on the product label. This study evaluated the effect of COVIDEX<sup>®</sup> on modulating immune response in laboratory mice following induction of inflammatory immune response similar to that observed in COVID-19 cases. The mice were given COVIDEX<sup>®</sup> orally at three dose levels daily for 28 days before induction of inflammation using Lipopolysaccharide administered by intraperitoneal route. After induction of inflammation, the mice spleens were harvested and splenocytes were processed for stimulation studies. COVIDEX<sup>®</sup> dose of 0.4 ml/kg daily was found to significantly increase the population percentage of CD4+ T cells in the treated group compared to the control ( $p=0.01$ ) and this effect was dose dependent. COVIDEX<sup>®</sup> also at doses of 0.1 ml/kg and 0.4 ml/kg significantly suppressed inflammation as indicated by the smaller value of CD4+/IL-10+ in the treated group compared to the control group ( $p=0.02$ ) and ( $p=0.02$ ) respectively. However, the percentage of natural killers reduced significantly ( $p<0.05$ ) at all three dose levels studied. This study indicates that COVIDEX<sup>®</sup>, is an immunomodulator that may be useful in preventing adverse immune response known as cytokine storm seen in COVID-19 patients.

**Key words:** Immunomodulation, herbal, product, COVIDEX<sup>®</sup>, COVID-19, berberine, alkaloids.

## INTRODUCTION

Immunomodulation is a broad term referring to any changes in the immune response that may involve

induction, expression, amplification, or inhibition of any part or phase in the immune response. The role played

by the immune system has become increasingly important in the understanding of the mechanisms involved in disease prevention majorly through innate and adoptive immune response (Samec et al., 2020; Behl et al., 2021).

The concept of immunomodulation has been gaining much significance worldwide as people start realizing the indispensable role of the immune system in disease prevention, control or cure. The control of disease by immunological means has two objectives: The development of immunity and the prevention of undesired immune reactions. Many agents of synthetic and natural origin have stimulatory, suppressive, and regulatory immune activities (Maheshwari et al., 2022; Nair et al., 2019).

Herbal medicines with immunomodulatory activity alter the immune function through the dynamic regulation of molecules such as cytokines and chemokines. Several plants are reported to exhibit immunomodulatory actions like phagocytosis promotion and macrophage activation, modulation of cytokine secretion, immunoglobulin production, allergic reactions, and lymphocyte proliferation (Nair et al., 2019; Safriani et al., 2022). There is a growing interest towards the use of herbal medicines as multi-component agents to modulate the complex immune system in the control of infections (Jantan et al., 2015).

Coronavirus Disease 2019 (COVID-19) caused by an infection with the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has caused one of the largest global outbreaks in recent years and posed a serious threat to global public health (Kumar and Al Khodor, 2020). One of the key features of COVID-19 is the overwhelming inflammation observed in some patients, especially those who develop severe illness. An exaggerated immune response (cytokine storm) mediated by an array of cytokines plays a role in the pathogenesis of the disease (Wong, 2021).

COVIDEX® an herbal product formulated from plant water extracts of *Zanthoxylum gillettii*, *Warburgia ugandensis* and *Cymbopogon citratus* was developed in Uganda and widely used during the SARSCOV-2 delta wave deadliest attack on the nation in 2021. The product approved by Uganda National Drug Authority as traditional medicine for supportive treatment of viral infections is still popular in Uganda and was used by 26.2% of COVID-19 hospitalized patients in two large COVID treatment centers in Uganda (Musoke et al., 2021). The product is formulated as a liquid for application into the mouth and released into the throat slowly to enable longer contact with the nasopharynx where SARSCOV-2 initially lodges and replicates itself.

The product label indicates that it contains 8.5% berberine in addition to other phytochemicals found in the medicinal plants used in its formulation. Berberine which is an isoquinoline alkaloid is reported to have antiviral effects against multiple isolates of SARS-CoV-2 (Varghese et al., 2021). In addition, some *Zanthoxylum* species have been reported to contain compounds that have antiviral activities on viruses such as influenza, picornaviruses, human immune deficiency virus, and herpes simplex among others (Okagu et al., 2021; Iloghalu et al., 2022; Shao et al., 2020). While COVIDEX® contains antiviral compounds such as berberine alkaloid and is being widely used in Uganda, its effect on the immune function remained unexplored. This study aimed to explore the immunomodulatory potential of COVIDEX herbal product to understand its other potential modes of action in addition to the antiviral effects of alkaloids such as berberine.

## METHODOLOGY

### Study site

Mbarara University of Science and Technology animal house facility and Makerere University College of Health Sciences immunology laboratory.

### Herbal product material

COVIDEX is a water based herbal preparation made by Jena Herbaria Limited from medicinal plants with antiviral and immunomodulatory activities that have long history of use in traditional medicine. The traditional medicinal plants used to make COVIDEX are *W. ugandensis*, *Z. gillettii* and *C. citratus*. COVIDEX contains berberine compound which is reported to exhibit various antiviral effects (Varghese et al., 2021; Babalghith et al., 2022). Combination of herbal preparations containing berberine (BBR) and obatoclox have also been recently reported to synergistically exhibit anti-SARS CoV-2 activity (Varghese et al., 2021). COVIDEX®, is trademarked by Uganda Registration Services Bureau and also approved by Uganda National Drug Authority as supported treatment in management of viral infections with registration number THA 928 (NDA, 2021) and is widely sold in pharmacies in Uganda.

### Materials and reagents

The materials and reagents used to prepare mouse splenic samples and acquire flow cytometry data were: 1 ml BD Syringes, 50 mL centrifuge tubes, 70 µm cell strainers, 10 mL serological pipette, cryogenic tubes, lipopolysaccharides (LPS), FACS tubes, pipettes, ice bucket, a centrifuge with brakes off, blood lysis buffer, 10% culture media, antibody cocktail, 10% complete media (CM), flow cytometry buffer, phosphate-buffered saline (PBS), fixation/permeabilization buffer, BD Perm/wash buffer, PMA/Ionomycin, and

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Brefeldin. LPS was purchased from Sigma-Aldrich (USA). The following antibodies in table 1: APC/Fire 750 anti-mouse CD8b.2, FITC- anti-mouse CD11b, APC- anti-mouse CD335, Pac Blue-anti-mouse CD4, PE-anti-mouse CD3, BV 785™- anti-mouse IL-2, BV605-anti-mouse IFN-g, PE/Dazzle 594-anti-mouse IL-10, were purchased from BioLegend (USA).

### Laboratory animals and the study drug

Twenty-four mice (24) adult male and female Swiss albino mice weighing between 20-30 g were obtained from Animal Facility Research, Mbarara University of Science and Technology, Uganda. The mice were fed on grower pellets and had free access to water. The mice were acclimatized for two weeks before the experiments. All the animals in the experimental study were treated humanely according to the Organization for Economic Co-operation and Development (OECD) guidelines. COVIDEX samples that were used in this study were donated by Jena Herbals Limited in Uganda (manufacturers and distributors) and some were purchased from SPELA Pharmacy, a National Drug Authority-licensed pharmacy in Mbarara City Uganda.

### Treatment of the animals

Twenty-four mice (24) were divided into four groups, each with six mice; mice in groups I – III were daily orally administered COVIDEX (0.1, 0.2, and 0.4 ml, respectively) once a day by oral cannula while the mice in group IV were given distilled (negative control) for 28 days. On the last day of treatment, mice in all the groups were administered 25 µg of lipopolysaccharide (LPS) by intraperitoneal route, and after 12 h of observation, all the mice were euthanized, and the spleen collected from each mouse under sterile conditions.

### Sample preparation of mouse splenic leucocytes

The spleens removed were put into tubes containing 30 ml of 1× PBS. Using a 1 ml syringe, each spleen was transferred to a 70 µm cell strainer on top of a 50 ml tube. Using the back of a syringe plunger, the cells (leucocytes) were macerated and passed through the filter. The filter was rinsed at regular intervals with 1× PBS. The tube was filled with cold 1× PBS and centrifuged at 300 × g at 2-8°C for 10 min. The supernatant was discarded, and the cells were then suspended in 5 ml 1× PBS and counted. After counting, splenocyte-containing tubes were filled with cold 1× PBS and centrifuged, the supernatant was discarded, and cells were resuspended in 1× PBS at a concentration of 1 × 10<sup>7</sup> cells/ml. The cells were stored under liquid nitrogen until Fluorescence-activated cell sorting (FACS) data acquisition.

### Splenocytes thawing

Cryopreserved splenocytes were retrieved from liquid nitrogen at -196°C and immediately transferred to a preset 37°C water bath. Upon thawing, cells were washed in a complete RPMI 1640 medium and incubated overnight at 37°C in 5% carbon dioxide (CO<sub>2</sub>). Thereafter, the cell yields and viability was determined using trypan blue solution. Samples with at least 70% viability were used for stimulation assays.

### FACS data acquisition

#### Cell surface staining

About 4.0 × 10<sup>5</sup> cells/mL were surface stained and incubated for 30 min with the following antibodies in table 1: APC/Fire 750-anti-mouse CD8b.2, FITC- anti-mouse CD11b, APC- anti-mouse CD335, Pac Blue-anti-mouse CD4, PE-anti-mouse CD3. 1 µL each of surface markers was added to the samples and incubated for 30 min at room temperature in the dark. The cells were washed twice by adding cell staining buffer and centrifuged at 1800 rpm for 5 min. The cells were acquired on a 19-color CytofleX flow Cytometer (Beckman coulter, New Jersey, USA). At least 100,000 events were recorded for analysis. Gating was standardized and set using fluorescence minus one control (FMOs) (Amanya et al., 2022).

#### Intracellular staining

After surface staining, the splenocytes were fixed by adding 0.5 mL of fixation buffer to each sample tube, incubated in the dark for 30 min at room temperature, and then centrifuged at 1800 rpm for 5 min and the supernatant was discarded. The fixed cells were permeabilized by re-suspending in intracellular staining BD perm/wash buffer (diluted to 1× in deionized water) and centrifuged at 1800 rpm for 5 min. A cocktail of intracellular cytokine antibodies was prepared by adding 1 µL of each, that is, Violet 785™ Brilliant anti-mouse IL-2, BV605-anti-mouse IFN-g and PE/Dazzle 594-anti-mouse IL-10 in the tube. The antibody cocktail was added to each of the sample tubes and incubated for 20 min in the dark at room temperature. Cells were washed 2 times with 2 mL of intracellular staining BD Perm/Wash Buffer and centrifuged at 1800 rpm for 5 min. The cells were then resuspended in 100 µL cell staining buffer and acquired on the Cytoflex LX flow cytometer. Compensations and fluorescent minus ones (FMOs) controls for some antibody fluorochromes were run together with the optimization gains for every channel using unstained cells. Compensation calculations were done and applied to the samples. At least one hundred thousand events were acquired and recorded per sample. Data was exported for analysis using flowJo software (Amanya et al., 2022).

### Statistical data analysis

Fluorescence-activated cell sorting (FACS) data was acquired using a flow cytometer (Cyto Flex) and analyzed using FlowJo software version 10.8.1. Lymphocytes were carefully gated on a forward scatter and side scatter plot. The percentage of positive cells for each marker was determined based on the number of viable lymphocytes. Bivariate dot plots or probability contour plots were generated upon data analysis to display the frequencies of and patterns by which individual cells co-express certain levels of cell surface antigen and intracellular cytokines. Statistical data were analyzed using Graph Pad Prism version 8.0.3 (Prism, 2018). Results obtained were presented as graphs. Differences in the frequencies of the innate and adaptive cells were evaluated using a One-way Analysis of Variance (ANOVA) followed by Dunnett's Multiple Comparison Test. P-values < 0.05 were considered to be statistically significant.

### Ethical consideration

All the animals in the study were handled humanely following Institutional Animal Care and Use Committee guidebook 2nd Edition 2002. Office of Laboratory Animal Welfare (OLAW) National

**Table 1.** Surface markers and intracellular cytokines staining panel used.

S/N	Fluorophore	Marker	Function
1	PE	CD3	Involved in activating both CD4 & cytotoxic T cells
2	Pac Blue	CD4	signals to other types of immune cells,
3	APC/Fire 750	CD8b.2	Kill virally infected cells
4	FITC	CD11b	Expressed on monocytes; regulates leukocyte adhesion and migration to mediate the inflammatory response
5	APC	CD335	Exclusively expressed on NK cells.
6	BV605	IFN- $\gamma$	Activate macrophages
7	BV 785 <sup>TM</sup>	IL-2	Promote cytotoxic T-cell proliferation
8	PE/Dazzle 594	IL-10	Regulation of T cell-mediated responses
9	Zombie aqua	Live/dead	Differentiate dead and live cells

Institutes of Health  
(<https://grants.nih.gov/grants/olaw/guidebook.pdf>).

one-way ANOVA and p-values less than 0.05 were taken to be statistically significant.

## RESULTS

### Effect of COVIDEX on CD4 T cell activation

The percentage of CD4+T cells (a), expressing IFN-g+ (b), IL-2+ (c), and IL-10 (d) for the control and treatment groups (n=6) is shown in Figure 1. The difference between the intervention and control group mean percentages above was determined using one-way ANOVA and p-values less than 0.05 were taken to be statistically significant.

### Effect of COVIDEX on CD8 T cell activation

The percentage of CD8+T cells (a), expressing IFN-g+ (b), IL-2+ (c), and IL-10 (d) for the control and treatment groups (n=6) is shown in Figure 2. The difference between the intervention and control group mean percentages above was determined using one-way ANOVA and p-values less than 0.05 were taken to be statistically significant.

### Effect of COVIDEX on innate immune responses

The percentages of monocytes (a) and NK cells (b) obtained following surface staining of mice splenocytes for the control and treatment groups (n=6) are shown in Figure 3. Following intracellular staining, the percentage of NK cells expressing IFN-g (cytokine) did not increase (c). The difference between the intervention and control group mean percentages above was determined using

## DISCUSSION

This study investigated the effect of COVIDEX on immune responses, specifically looking at the CD4 and CD8 T cell activation and their cytokine expression levels in a mouse model of lipopolysaccharide (LPS) induced inflammation. The results revealed COVIDEX has immunomodulatory and selective effects on distinct immune cell populations.

Similar observations of immune modulation by herbal drugs have been reported in previous studies. For instance, herbal extracts have been found to enhance the proliferation and activation of T lymphocytes, including CD4 T cells, thereby promoting immunomodulatory effects (Alhazmi et al., 2021; Alanazi et al., 2023).

This study found that the percentage population of CD4+T cells increased in the COVIDEX treated group and the effect was dose dependent becoming statistically significant at dose of 0.4 ml/kg (Figure 1a). The increase in population of CD4+T cells is potentially beneficial in treatment of diseases that suppress CD4+T cells as seen in Human Immunodeficiency Virus (HIV) patients. This study also revealed that COVIDEX caused a dose dependent but non-significant increase in IFN-g and IL-2 expression levels by CD4 T cells after COVIDEX treatment, suggesting it doesn't directly significantly affect the production of these pro-inflammatory cytokines under the conditions tested (Figure 1b and c). Interestingly, the study found that COVIDEX treatment significantly reduced the expression of IL-10 by CD4 T cells (Figure 1d), a key immunosuppressive cytokine involved in regulating inflammatory responses, limiting unnecessary tissue injuries (Saraiva et al., 2019). The decrease in IL-

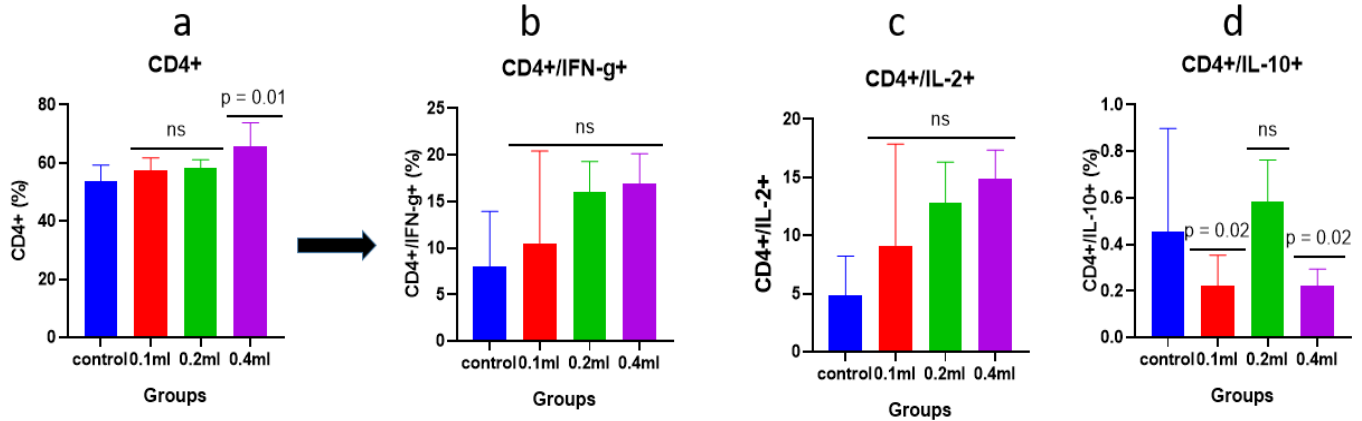


Figure 1. Expression of IFN-g+, IL-2+, and IL-10 by activated CD4+ T cells.

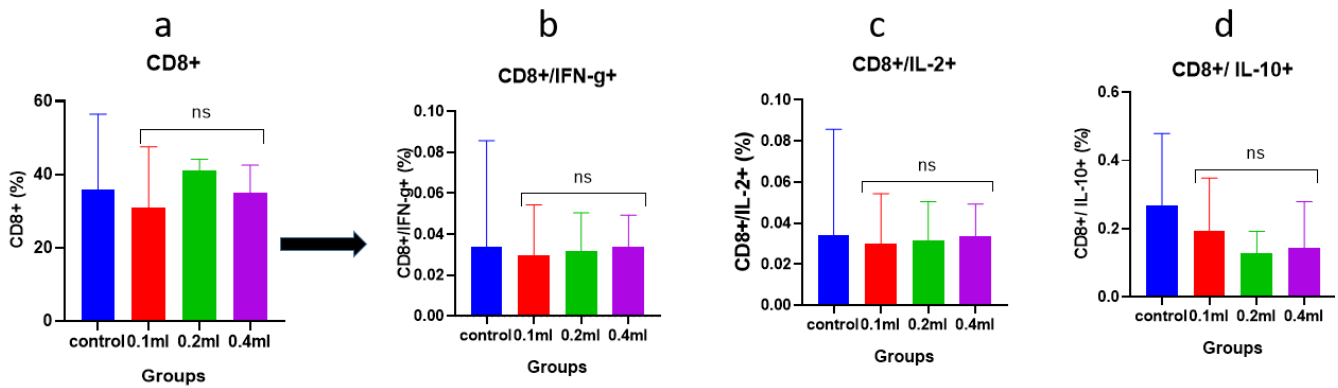
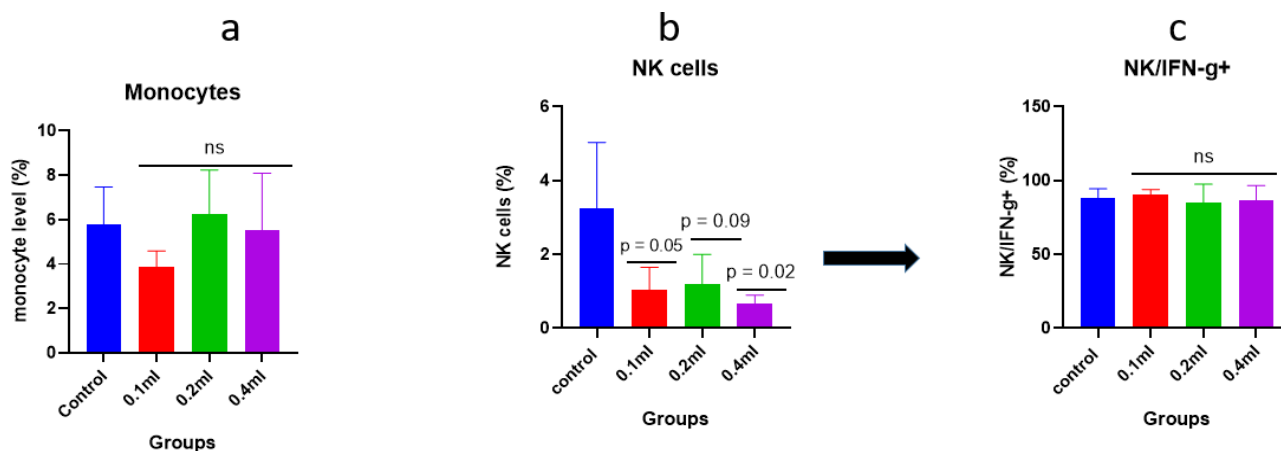


Figure 2. Expression of IFN-g+, IL-2+, and IL-10+ by activated CD8+ T cells.

10 expression indicates that COVIDEX may have anti-inflammatory properties by potentially downregulating the production of this immunosuppressive cytokine. This finding aligns with previous studies demonstrating the anti-inflammatory effects of various herbal compounds (Mollazadeh et al., 2019, Ghasemian et al., 2016). Similarly, we investigated the impact of COVIDEX treatment on CD8 T cells (Figure 2a). These findings show that it did not significantly affect the proportion of CD8 T cells or their cytokine (IFN-g, IL-2, IL-10, and IFN-g) expression in mice with LPS-induced inflammation (Figure 2b, c, and d). These results suggest that COVIDEX's impact on CD4 T cells may be more pronounced than on CD8 T cells. This is evidenced by the observed changes in CD4 cell percentages and IL-10 expression, highlighting its complex immune modulation and selective effects on distinct immune cell populations. This is an important observation that demonstrates that COVIDEX antiviral activity may also be through cytotoxic

mechanisms (Singh et al., 2021).

This study investigated the effect of COVIDEX on innate immune cell populations, particularly monocytes and NK cells, and their response to LPS-induced inflammation. The findings indicate that COVIDEX did not significantly affect monocytes (Figure 3a). However, it led to a decrease in the percentage of NK cells (Figure 3b). Interestingly, despite this decrease in NK cell percentage, there was no significant difference observed in the expression of IFN-gamma (IFN-g) by NK cells between the control and treatment groups (Figure 3c). These findings suggest that while COVIDEX may not directly affect monocyte populations, it seems to affect NK cells, reducing their numbers and inhibiting their functional ability to produce IFN-g. Lipopolysaccharide (LPS), a key component of Gram-negative bacteria cell walls, triggers an inflammatory response in monocytes, causing them to infiltrate tissues and adopt inflammatory macrophage and dendritic cell (DC)-like phenotypes to fulfill their effect or



**Figure 3.** Activated monocytes and expression of IFN-g by activated NK cells

functions of pro and anti-inflammatory activities (Tucureanu et al., 2018; Knoll et al., 2021). The findings suggest that COVIDEX may have a specific mechanism or receptor interaction that selectively affects NK cells, but not monocytes. Natural Killer cells possess a distinct ability to recognize and fight viruses by performing cytolytic activity against infected cells and by producing antiviral cytokines. IFN-g affects viral replication and entry into host cells, as well as recruiting and activating other effector leukocytes, including cytotoxic T lymphocytes and CD4+T helper type 1 cells (Al-Ani et al., 2020). Therefore, our findings suggest that COVIDEX exhibits anti-inflammatory properties by reducing NK cell populations and inhibiting their IFN-g production. COVIDEX effects appear complex consisting of antiinflammation as well as direct antiviral effects through the alkaloids and other compounds in it not involving mechanism of controlling viral infections through NK cell-mediated killing (Björkström et al., 2022). This is because COVIDEX contains Berberine, a well-known phytochemical that, exhibits significant antiviral properties against a wide range of viruses (Valipour et al., 2023). This phytochemical has been investigated, by several studies, as a potential COVID-19 adjunct therapy due to its anti-inflammatory, antioxidant, antiviral, and immunoregulatory properties (Babalghith et al., 2022).

## Conclusion

COVIDEX's ability to modulate specific IL-10-expressing CD4 T cells suggests its potential as a therapeutic agent for conditions involving dysregulated immune responses such as COVID-19 infection. Its ability to increase CD4+ T cell population without increasing CD8+ T cell population also makes it a potentially useful product in chronic viral infections such as HIV however, the reduction in the

percentage of NK cells needs further investigation since this could be due to increase in population of CD4+ T cells without corresponding increase in population of NK cells.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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