Original Article

Immunomodulatory effect of a proprietary polyherbal formulation on healthy participants: A single- blind, randomized, placebo- controlled, exploratory clinical study

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Abstract

Context: Clinical study for immunity.

Aims: The present study aimed to assess the effect of proprietary polyherbal formulation (PPHF), labelled as Kofol immunity tablets (KIT) on innate and adaptive immune responses in healthy individuals, on the backdrop of COVID-19 pandemic.

Settings and Design: Single-blind, randomized, placebo-controlled, exploratory study in institutional setting.

Materials and Methods: Post Ethics Committee permission, screened healthy individuals of either sex aged 18–35 years were randomized to PPHF/Placebo for 2 months. Major assessment variables included peak expiratory flow rate (PEFR), questionnaire-based immune status, perceived stress, and quality of life (QOL) with immune-specific cell counts (CD4+, CD8+), cytokines (interferon gamma [IFN-γ], tumor necrosis factor-alpha [TNF-α], interleukin 10 [IL-10]), and oxidative stress in red blood cells (RBCs) (malondialdehyde (MDA), glutathione peroxidase [GPx]), done at day 60.

Statistical Analysis Used: Mean ± standard deviation and paired/unpaired *t*-test for parametric data analysis while median (range) and Wilcoxon Rank sum test/Mann–Whitney test for nonparametric data analysis, were done. Categorical data was analyzed using Chi-square test. GraphPad InStat software, version 9 was used with $p < 0.05$, as the level of statistical significance.

Results: Of 52 recruited, 28 individuals completed the study. PPHF significantly increased PEFR, improved immune status along with QOL compared to baseline. It also decreased perceived stress from moderate and severe grade to mild. Serum IFN-γ levels remained almost constant post-PPHF treatment. PPHF significantly decreased MDA and increased GPx in RBCs. Significant decrease and increase in TNF-α and IL-10, respectively, were seen in PPHF group. The safety parameters post-PPHF treatment remained within normal reference ranges.

Conclusions: PPHF is an efficacious and safe formulation with immunomodulatory potential.

Keywords: CD4-CD8 counts, COVID, immunity, inflammation, oxidative stress, peak expiratory flow rate

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INTRODUCTION

Outbreak of COVID-19 pandemic has highlighted the importance of a well-functioning immune system. Immune modulation or immune boosting have become popular words since the pandemic era and are being used erroneously. The herbal drug market is flooded with patent and proprietary formulations claiming to be immune boosters. The fear and anxiety of the pandemic in the community coupled with vaccines unavailability during the 1st wave was the major reason behind the upsurge in manufacture and sale of these formulations. However, many of these formulations lack credible evidence that is required to rationalize their use. The claims about these formulations are primarily based on the reported pharmacological activities of individual ingredients.

Charak Pharma Private Limited introduced a patent and proprietary Ayurvedic formulation, kofol immunity tablets (KIT) during the pandemic for improving immunity. It is composed of medicinal plants such as *Tinospora cordifolia*, *Curcuma longa, Zingiber officinale,* and *Piper longum* each having proven antioxidant and anti-inflammatory activities.[1-7] Further, these plants are reported to stimulate the immune system and are considered to alter immune response through the dynamic regulation of cytokine secretion.[8]

The present study was therefore planned to generate scientific evidence for this formulation to assess its effect on immune responses in healthy individuals in the face of the pandemic. The immune responses involve secretion and activation of various cells such as dendritic cells, macrophages, neutrophils, natural killer cells, B-cells and T-cells, cytokines like tumor necrosis factors, interleukin (IL), interferons, and protein molecules.[9] Further, psychological stress is also known to cause decreased responsiveness of the immune system.[10] It has also been validated that oxidative damage (ROS production) and reduced antioxidant potential can hamper efficiency of immune system.[11,12] We, therefore, selected parameters covering these various aspects.

MATERIALS AND METHODS

Study design and setting

It was a single-blind, randomized, placebo-controlled, exploratory clinical study conducted from January 2021 to November 2021. The participants were blinded in the study. The recruitment was on hold during March–April 2021 due to the lockdown imposed in view of 2nd wave of COVID-19. It was resumed postlockdown.

Ethical consideration

The study was approved by Institutional Ethics Committee (BVDUCOA/EC/2829/2020-2021) and registered prospectively with Clinical Trial Registry of India (CTRI/2020/12/030139). It was conducted according to Good Clinical Practices and in compliance with the Declaration of Helsinki. A written informed consent was obtained from all participating individuals before start of the study.

Sample size

As it was an exploratory study, a sample size of 30 completed participants (20 in PPHF group and 10 in placebo group) was considered adequate.

Eligibility criteria

Inclusion criteria

Young age adults, without any known comorbidities, aged 18–35 years of either sex were considered for screening and called to the study site (day -3). Their health status was confirmed using blood investigations namely hemogram (hemoglobin [Hb], white blood cells [WBC count], erythrocyte sedimentation rate, platelets), fasting blood glucose, liver function tests (LFT) (serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase), and renal function test (RFT) (serum creatinine).

Exclusion criteria

Individuals with Hb levels ≤9 g%, LFT, and RFT levels >2.5 times and 1.5 times, the upper normal limit, respectively, were excluded from the study. Besides, those with a history of any thyroid, cardiac, liver, or renal pathologies were also excluded from the study. Individuals with a history of consumption of herbal/nutritional/pre and probiotic supplements/multivitamins/or available marketed medications for immunity for the past 30 days were also not included. Lactating and pregnant women were not considered for the study.

Study intervention and dosage

Both interventional drugs, PPHF and placebo were supplied by Charak Pharma Private Limited.

Each tablet of the proprietary polyherbal formulation, weighing 618 mg consisted of Triphala Guggul (Ayurveda formulation; 200 mg), and aqueous extracts derived from medicinal plants namely Guduchi (*Tinospora cordifolia*), Haridra (*Curcuma longa*) (500 mg each), Manjishtha (*Rubia cordifolia* 250 mg), Chitrak (*Plumbago zeylanica* 150 mg), and Trikatu (a combination of *Zingiber officinale, Piper nigrum, Piper longum;* 66.66 mg each) in addition to excipients

while placebo was composed of maize starch as the main ingredient along with other constituents. Both PPHF and Placebo were administered in the dose, 1 tablet twice a day after meals with water for 2 months.

Methodology

As it was an exploratory study, the actual sample size considered for the study was 30. Considering the 20% attrition rate, 36 individuals were recruited, of which 30 were expected to complete the study. These recruitments happened in the month of January–February 21 when there were very few active cases of COVID-19. The follow-up of these participants fell during the second wave of COVID-19 (March–April 2021), due to which almost 50% of participants dropped out. In order to attain the planned sample size, additional screening and recruitments were done with permission of the Institutional Ethics Commitee. The recruitment of the participants was resumed in June 2021 (i.e., after decline in number of cases). Written informed consent from all the participants was thus obtained in person.

Once eligible, the participants were called to the study site within the next 3 days for baseline visit (day 0) and examined for vitals (temperature, pulse, and blood pressure), respiratory health (respiratory rate, oxygen saturation [SpO_2], and peak expiratory flow rate [PEFR]). Their immune status, perceived stress, and quality of life (QOL) were assessed using standardized and validated questionnaires, namely immune status questionnaire (ISQ),[13] perceived stress scale 10 (PSS-10),[14] and World Health Organization QOL (WHOQOL-BREF).[15]

This was followed by blood collection (12 ml) for the estimation of immune and oxidative stress parameters. Subsequently, the participants were randomized by chit method to two groups namely PPHF and Placebo in the ratio of 2:1. As evaluation of the effect of the study drug on immunity was the main aim of the study, a greater number of participants were considered in the study group.

The study interventions were provided for 30 days. On day 30, they were provided medications for further 30 days and called on day 60. All assessments done at baseline were repeated on day 60 along with hemogram, LFT, and RFT (which were done at screening visit). Participants were instructed to report adverse events, if any, throughout the study period.

Additional measures during COVID-19

Considering the risk of COVID-19, all safety precautions such as wearing a mask, regular sanitization of the study site, and maintenance of safe distance were observed. Individuals with a complete absence of any flu-like symptoms were only approached for screening and they were also assessed by the study physician. All aseptic precautions were taken during blood collection and processing. The instruments used for collection of data; thermometer for temperature, digital monitor for pulse and blood pressure, pulse oximeter for SpO_2, and peak flow meter for PEFR were thoroughly sanitized prior and post use of every participant. Telephonic follow-ups were maintained regularly to enquire about the health of participants.

Blood sampling and processing

A closed collection procedure was adopted while collecting blood to minimize contamination. Of 12 ml of blood collected at baseline, 1.5 ml was processed for flow cytometry-based estimation of immunological surface markers (CD4+ and CD8+). From 9 ml blood, PBMCs and red blood cells (RBCs) were isolated, and rest 1.5 ml was centrifuged to obtain serum for estimating serum interferon-gamma (IFN-γ). On day 60, additional 3 ml blood (total 15 ml) was collected for the estimation of parameters done at screening visit except fasting glucose.

Cytokine estimation from stimulated PBMCs

Above collected 9 ml blood was subjected to density gradient centrifugation using Histopaque (1:2 in phosphate-buffered saline) to separate PBMCs and RBCs. Isolated PBMCs in the density 5×10^5 cells were cultured in each well and seeded in RPMI 1640 medium for 2 h. Later, the cells were stimulated with *Escherichia coli* derived lipopolysaccharide (LPS) at 0.5μg/ml concentration and incubated for 4 h, at 37°C in 5% CO_2 incubator. The culture supernatant was harvested for the estimation of cytokines namely tumor necrosis factor-alpha (TNF-α) and IL-10 using commercially available enzyme-linked immunoassay kits (BioLegend).

Oxidative stress estimation from red blood cells

Malondialdehyde (MDA) was quantified from RBCs using thiobarbituric acid reactive substances method.[16] Glutathione peroxidase (GPx) levels from the RBCs lysate (1:10 diluted in phosphate buffer) were measured using commercially available colorimetric assay kit (Cayman, US).

Statistical analysis

Parametric data has been presented as mean ± standard deviation and analyzed by paired/unpaired *t* test. The nonparametric data has been presented as median (range) and analyzed by Wilcoxon Rank sum test/Mann–Whitney test Categorical data was analyzed using Chi-square test. GraphPad InStat software, version 9 (Graphstats

Tecnologies Pvt. Ltd., Bangalore, Karnataka, India) was used for data analysis with $p < 0.05$, as the level of statistical significance.

RESULTS

A total of 67 individuals were screened, of which 52 healthy individuals were recruited; 36 in the PPHF group and 16 in the placebo group. Of these, 28 participants completed the study; 18 from PPHF group and 10 from placebo group. There were 15 dropouts and 3 withdrawals in PPHF group, while 6 dropouts in placebo group. Majority of dropouts (irrespective of the study group) occurred during the lockdown period (March–April 2021).

There were two participants who had reported flu-like symptoms during the study in the PPHF group and tested positive for COVID-19 through reverse transcription polymerase chain reaction.

The participant flow is given in Figure 1.

Of 18 completed participants in PPHF group, there were 10 males and 8 females while among the 10 completed participants in placebo group, there were 7 males and 3 females. The mean age of all completed participants in both groups was comparable; 25.9 ± 5 years in PPHF group and 24.3 ± 5.4 years in placebo group.

Effect of PPHF on vitals

There were some minor changes in vital parameters namely temperature, pulse, and blood pressure in both the groups, which were not significant statistically or clinically. Among respiratory health parameters too, there were minor and nonsignificant variations in respiratory rate and SpO_2. A significant ($p = 0.0385$) increase in PEFR was seen in PPHF group on day 60 compared to day 0, suggestive of improved lung capacity. There was no such increase in placebo group.

Effect of PPHF on subjective assessments

When the effect of PPHF on immunity was evaluated subjectively using ISQ, the immune status score was found significantly ($p = 0.0001$) increased in PPHF group on day 60 compared to day 0. It remained significantly ($p = 0.0071$) higher than placebo group.

Stress in participants was evaluated using PSS-10 and scores were divided into categories namely mild, moderate, and severe. It was seen that 1 participant each from moderate and severe stress category in PPHF group changed to mild stress category post treatment while in placebo group, 1 participant from mild stress category changed to severe stress category, though none of these changes were significant.

The effect of PPHF on QOL was assessed using WHOQOL-BREF. An increase in QOL scores was seen only in 1 domain (physical) out of 4 domains in placebo group, whereas in PPHF group, an increase was noticed in 3 domains except for psychological domain. The increase in both PPHF and placebo groups was not statistically significant.

Effect of PPHF on CD4 and CD8 cell counts

An increase was seen in CD4 count in PPHF group on day 60 while it decreased in placebo group compared to day

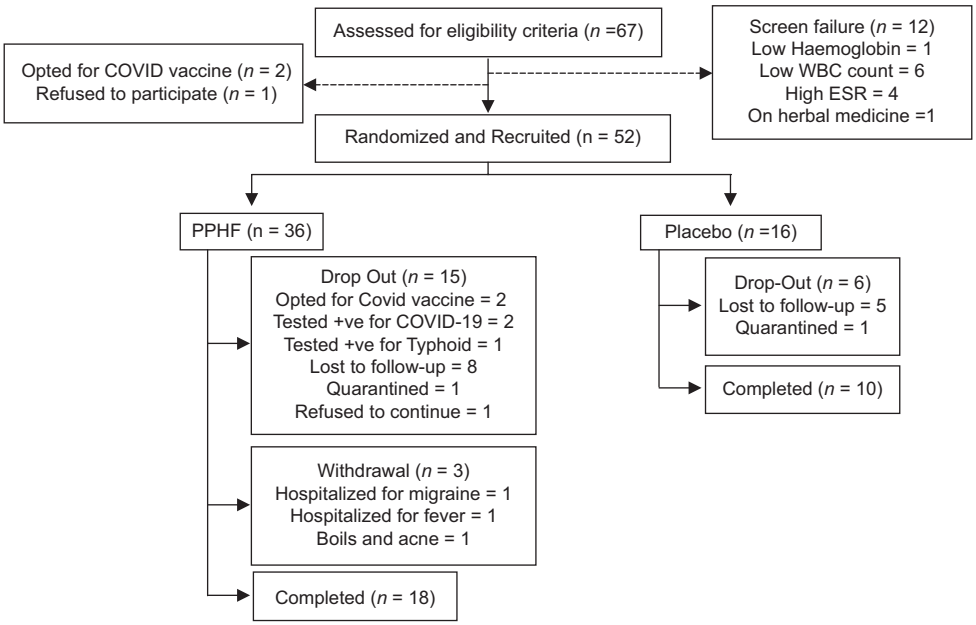

Figure 1: Flowchart of study participants

0. There was a significant ($p = 0.0044$) difference in CD4 counts between PPHF and placebo group on day 60. The CD8 counts remained almost constant in PPHF group while they significantly ($p = 0.0195$) decreased in placebo group on day 60 compared to day 0. The CD8 counts in the placebo group on day 60 were significantly ($p = 0.0452$) lesser than the PPHF group. All these observed values in both groups were within the normal range of the laboratory [Table 1].

Effect of PPHF on oxidative stress

MDA from RBCs in PPHF group showed a significant ($p = 0.0067$) decrease post treatment compared to day 0; while in placebo group, there was an increase in MDA levels on day 60 compared to day 0. There was an increase in median GPx levels from RBCs in both, PPHF and Placebo groups on day 60 compared to day 0, that was significant ($p = 0.0129$) only in PPHF group [Figure 2].

Effect of PPHF on cytokines

Median levels of IFN-γ in PPHF group after treatment were almost similar to baseline while a slight increase was seen in placebo group on day 60 compared to day 0.

In PBMCs treated with LPS, PPHF group demonstrated significant decrease ($p = 0.0009$) in TNF-α (a pro-inflammatory cytokine) on day 60 compared to day 0 while in placebo group the levels remained almost similar. IL-10 (anti-inflammatory cytokine) levels in LPS stimulated PBMCs significantly ($p = 0.0003$) increased in PPHF group while in placebo group, they were almost constant on day 60 compared to day 0 [Figure 3].

Effect of PPHF on safety parameters

The total WBC count in PPHF group significantly ($p = 0.039$) increased on day 60 compared to day -3 (screening). No such increase was seen in placebo group. Although the values were within the normal range, the difference between PPHF and placebo groups was statistically significant. In case of the differential count of WBCs, the lymphocyte count in placebo group was significantly higher ($p = 0.006$) than PPHF on day -3 (screening). No such difference was noted on day 60, indicative of better lymphocyte recruitment in PPHF group than placebo. Other parameters though showed minor fluctuations were within normal range in both groups suggesting safety of PPHF [Table 2].

DISCUSSION

The present clinical study aimed to evaluate the immunomodulatory effect of proprietary polyherbal formulation (PPHF) in healthy individuals. The formulation significantly improved respiratory health and immune status of participants. There was a decrease in psychological stress along with improved QOL in 3 domains. The formulation maintained serum IFN-γ levels after treatment and significantly decreased oxidative stress (MDA) of RBCs. Furthermore, it significantly reduced inflammation by

Table 1: Effect of PPHF on different parameters

Parameters	PPHF (*n*=18)		Placebo (*n*=10)	
	Day 0	Day 60	Day 0	Day 60
Vitals				
Temperature (°C)	36.3±0.1	36.2±0.2	36.3±0.2	36.3±0.1
Pulse (/min)	77.7±7.5	77.8±7.7	83.3±8.5	82.2±7.8
Systolic pressure (mmHg)	111.6±14.2	116.9±10.3	117.8±16.7	120.2±21.2
Diastolic pressure (mmHg)	77.8±15.5	80.6±6.9	78.7±17.4	79.6±13.7
Respiratory health				
Respiratory rate (/min)	19.7±1.9	19.6±1.9	20.8±2.1	21.7±1.8
SpO_2 (%)	97.1±1.6	97.7±0.9	96.4±1.6	96.9±0.9
PEFR (L/min)	298.2±78.7	337±50*	290.9±115.9	297.7±131.2
Subjective assessment				
Immune status	8 (6-10)	10 (7-10)*,#	7.5 (6-10)	8 (5-10)
Perceived stress (*n*)				
Mild	3	5	5	4
Moderate	12	11	4	4
Severe	1	0	1	2
Quality of life				
Physical health	69 (56-100)	78 (63-99)	75 (31-86)	84.5 (44-100)
Psychological health	75 (56-96)	72 (44-94)	81 (24-89)	81 (44-86)
Social relationship	70 (31-94)	81 (50-94)	78 (15-100)	72 (40-96)
Environment	75 (56-89)	80 (63-96)	78 (31-94)	75.5 (19-90)
Cell count (cells/μL)				
CD4 count	929 (489-1503)	1043.5 (424-1687)##	976 (666-1268)	831.5(191-1089)
CD8 count	753 (347-1657)	728.5 (347-1422)#	930 (372-1260)	486 (164-1014)*

*$p<0.05$ as compared to day 0 using paired *t*-test/Wilcoxon rank sum test, #$p<0.05$, ##$p<0.01$ compared to placebo on day 60 using Mann-Whitney test. PEFR=Peak expiratory flow rate, SpO_2=Oxygen saturation

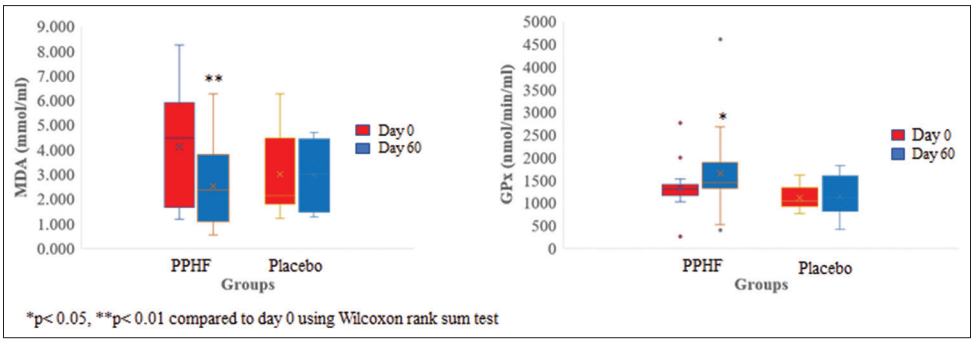

Figure 2: Effect of PPHF on oxidative stress

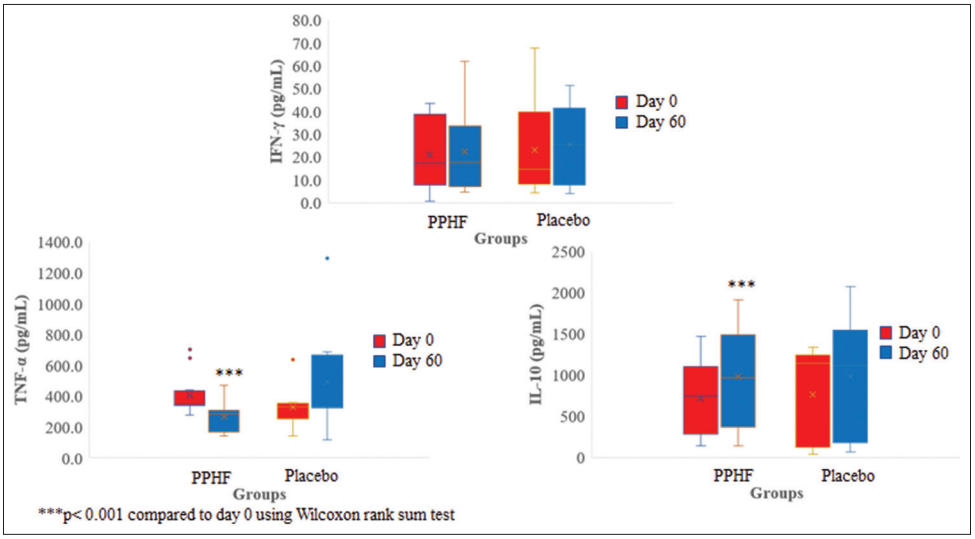

Figure 3: Effect of PPHF on cytokines

Table 2: Effect of PPHF on safety parameters

Parameters	PPHF (*n*=18)		Placebo (*n*=10)	
	Screening (day -3)	Day 60	Screening (day -3)	Day 60
Haemogram				
Hb (g/dL)	13.6±1.8	13.7±1.9	14.3±1.8	14.2±1.8
WBC (/cmm)	6977.78±1285.92	7729.41±1953.45*,#	6670±1352.41	6440±1145.23
Neutrophils (%)	60.72±5.17	59.24±7.61	57±5.46	56.5±8.04
Eosinophils (%)	2.78±1.11	2.65±1.50	2.5±1.08	2.6±0.7
Basophils (%)	0	0	0	0
Monocytes (%)	3.89±1.18	4.12±1.32	3.7±1.16	4.4±0.7
Lymphocytes (%)	32.61±4.46##	34±5.99	37.7±4.24	36.5±7.68
ESR (mm/h)	9.4±3.8	8.6±3.2	7.7±2.4	6.5±2.8
Platelets (10^3/cmm)	279±60.4	266±66.8	275.1±75.6	271±85.2
LFT (IU/mL)				
SGOT	17.5 (10-34.6)	21 (10-48)	15 (12-28)	17.5 (10-26)
SGPT	17.5 (8-67.2)	23 (8-82)	14.5 (10-36)	13 (10-44)
RFT (mg/dL)				
Serum creatinine	0.75 (0.5-1)	0.8 (0.5-1.1)	0.9 (0.6-1.1)	0.7 (0.6-1.4)

*$p<0.05$ compared to day-3 using paired *t*-test, #$p<0.05$, ##$p<0.01$ compared to placebo using unpaired *t*-test. Hb=Hemoglobin, WBC=White blood cell, LFT=Liver function test, RFT=Renal function test, SGOT=Serum glutamic oxaloacetic transaminase, SGPT=Serum glutamic pyruvic transaminase, ESR=Erythrocyte sedimentation rate

decreasing TNF-α and increasing IL-10. It was found to be safe throughout the study.

The Proprietary poly-herbal formulation, kofol immunity tablets (KIT) consists of medicinal plants with known anti-oxidant and anti-inflammatory activities. In the present study, we evaluated its effect on immunity as well as few other parameters important from the COVID-19 perspective. PEFR is an indicator of lung capacity.[17] The study began during the first wave of the pandemic

and there are reports about decreased lung capacity in SARS-CoV-2-infected individuals.[18,19] A significant increase in PEFR after PPHF administration, seen in our study participants is therefore noteworthy.

The effect of the formulation was further assessed subjectively on immune status, perceived stress, and QOL. ISQ is used to understand perceived immune status. A significant improvement was seen in PPHF group post treatment indicates self-satisfaction about own health. The profound effect of physiological factors like stress on immune system is well known.[12] Besides, a moderate level of stress impacting the mental health of the general population during the quarantine period of COVID is reported.[20,21] Psycho neuroendocrine immune (PNEI) axis links psychological and physical health well.[22] Hence, we evaluated the effect of the formulation on stress using PSS-10. The formulation reduced the stress as evident from change in stress category. Further, there are reports on decreased QOL during pandemic.[23,24] In our study, the formulation improved scores in 3 domains of QOL compared to only 1 domain in placebo, pointing toward better QOL in the formulation treated group, despite social crisis like COVID.

We also evaluated the effect of the formulation on different cells involved in immune responses. CD4+ T cells (T helper cells) are involved in the elimination of pathogens as part of adaptive immune response along with regulation of the cytokine secretion.[25] These cells are also critical for the generation of high-affinity memory B cells, long-lived plasma cells, and memory CD8+ T cells.[26] On the other hand, CD8+ cells are surface markers for cytotoxic T lymphocytes which are critically important in cell-mediated immunity to confront virally infected cells through endogenous antigen presentation, making them essential for maintaining protective immunity to many classes of infectious pathogens.[27] Further, effects of psychological stress on immune functions have been observed, which include decreased percentages of CD4 helper T cells and CD8 cytotoxic T cells with other factors.[28] In this study, CD4+ count increased in PPHF group post treatment, while CD8+ count significantly dropped in the placebo group compared to baseline.

IFN-γ is primarily released on the activation of CD4+ cells and is chief mediator of innate and adaptive immune response.[29] IFN-γ levels were almost constant in PPHF group after treatment, while they increased in placebo group post treatment. The mild variations observed in IFN-γ levels in both PPHF and placebo groups can be considered normal, as the participants were included in the study post confirmation of their health status.

TNF-α is a pro-inflammatory cytokine released in pathogenic conditions, indicative of high inflammation when increased.[30] On the contrary, IL-10, an anti-inflammatory cytokine, acts as antagonist inhibiting the production of pro-inflammatory cytokines such as IFN-γ and TNF-α.[31,32] Our results show significantly decreased levels of TNF-α from LPS-stimulated PBMCs in PPHF group on day 60 suggesting decreased inflammation in cells, while in the placebo group, almost constant levels of TNF-α on day 60, are indicative of sustained inflammatory response. IL-10 levels increased significantly in PPHF group while they were almost constant in placebo. It is possible that by immune regulatory action, IL-10 did not allow elevation of TNF-α level in the PPHF group on day 60.[33]

Generation of reactive oxygen species (ROS) can lead to lipid peroxidation inside cells and form products like MDA, quantified to determine the extent of cell oxidative damage.[34] As per studies, compromised immune functioning can increase ROS production in RBCs and oxidative stress inside cells affecting cell integrity, membrane damage, and hemolysis of RBCs.[11] Thus, MDA of biological membranes is a crucial indicator of oxidative stress. A significant reduction of MDA post treatment by the formulation indicates its potential to reduce oxidative stress in RBCs. This effect was not seen in placebo group. On the other hand, while free radicals deteriorate the immune system, antioxidants play an important part in cell defense mechanisms. GPx, an integral part of antioxidant system, protects cells from oxidative damage.[35] Its effect in modulating processes such as normal cellular growth, proliferative responses, and apoptosis are well studied. It is also involved in modulating pro-inflammatory responses.[36] In this study, though both PPHF and placebo showed an increase in GPx levels post treatment, it was statistically significant only in PPHF group. However, these results are inconclusive, in the absence of an estimation of reduced glutathione (GSH) that is known to play an important part in glutathione equilibrium.

There were reports of adverse events such as dyspepsia, heartburn by 2 participants in PPHF group during the study. However, there were no derangements in hepatic or renal function parameters in any of the participants post treatment in PPHF group, indicative of its safety.

In short, our results adequately demonstrated the immunomodulatory effect of the proprietary polyherbal

formulation in a small sample size. However, to generalize these findings, the study needs to be carried out in a larger sample size. Further, in-depth investigations to probe the effect on different components of the immune system can also be considered.

CONCLUSIONS

Polyherbal formulation when administered for 2 months to healthy individuals, caused improvement in their respiratory health, immune status, psychological stress, and QOL. It also reduced oxidative stress in RBCs, showed anti-inflammatory effect on LPS-stimulated PBMCs and was found to be safe. The proprietary polyherbal formulation, Kofol Immunity tablets can thus be considered a promising formulation with immunomodulatory potential.

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Conflicts of interest

Manisha Mishra, listed as co-author in this study represents the sponsor Charak Pharma Pvt. Ltd. She did not participate in the study conduct or analysis. Thus, there is no conflict of interest.

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