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Adaptogenic/Antistress Activity of a Polyherbal Formulation (Phytocee^{TM}): Mechanism to Combat Stress

Adaptogenic/Antistress Activity of Polyherbal Formulation

Divya Purusothaman[1], Sasikumar Murugan[1], Bharathi Bethapudi[1*], Prashanth D'souza[2], Deepak Mundkinajeddu[3]
[1]Department of Pharmacology, [2]Department of Animal health science, [3]Department of Biology, R&D centre, Natural Remedies Private Limited, Bengaluru, Karnataka, India.

Abstract:- It is inevitable for living beings to be away from stress. Chronic stress remains an etiology and also risk factor for progression of several pathological conditions. In this consideration to manage stress, adaptogens are employed. The present investigation is aimed to evaluate adaptogenic/antistress activity of a novel proprietary polyherbal formulation (PHF) in rodents and its plausible anti-stress mechanisms were identified in *in vitro* cell assays. Rats were exposed to chronic variable stress paradigm for sixteen days and PHF was administered concurrently at various dose levels. Immobility time of rats during forced swim test and serum cortisol levels were measured. Additionally adaptogenic activity of PHF to stress was evaluated in mice using swim endurance model with pre-treatment for ten days. To understand the defending mechanism, PHF was tested for corticotropin releasing hormone receptor 1 (CRHR1) antagonistic activity in Chinese hamster ovary-K1 (CHO-K1) cells. Also, effect on cortisol release and mRNA expression of 11β-hydroxysteroid dehydrogenase type 1 (*11β-HSD1*) against forskolin (10 µM) stimulated human adrenocarcinoma (H295R) cell were analyzed. PHF at all dose levels was found to decrease immobility time significantly during FST and reduced serum cortisol significantly at 15 mg/kg when compared to stressed group. PHF treatment was found to enhance swimming time significantly upto ~1.7 fold during swim endurance test when compared to untreated group. PHF (100 µg/mL) showed antagonistic effect of 26 % against CRF induced CRHR1 activity. Also, PHF (40 µg/mL) showed inhibition of cortisol release by 90.31 % and downregulated *11β- HSD1* expression by 11.5 fold in forskolin stimulated H295R cells. PHF demonstrated adaptogenic/antistress activity against chronic stress by its antagonistic effect on CRHR1, inhibition of cortisol release and downregulation of *11β-HSD1* expression.

Keywords:- *Anti-Fatigue, Physical Endurance, Chronic Variable Stress, CRHR1, Phytocee, 11β-HSD1 Expression.*

I. INTRODUCTION

Stress is defined as body's response to any sort of demand/threat imposed on it that disturb the normal physiological equilibrium. Acute stress are usually healthy that helps to be energetic, focused and productive. But chronic stress beyond the threshold cause major damage to the health, majorly compromises immune system leading to several ailments [1]. In this modern world, it is unavoidable for every population to be away from the stress. Like human, livestock also undergo stress due to climatic changes [2], housing, stocking and transportation etc. [3] that affects its productivity. It is of high importance to defend the negative impact of stress and to restore body's natural homeostasis. In management of stress, a special class of herbs called adaptogens gained considerable attention. Adaptogens have stabilization influence on physiological process. Unlike conventional chemicals that are specific to disease or symptoms, adaptogens, the natural defense enhancer create nonspecific resistance to adverse biological, chemical and physical stress. They are mostly meant to be prophylactic, boosting energy levels and create resistance to stress [4].

Keeping in view the impact of stress and the great demand for effective supplement to overcome stress and its consequence, the study was focused to evaluate the adaptogenic/anti-stress of a proprietary polyherbal formulation (PHF) and to understand its mechanism of action. The formulation includes three herbs viz. *Emblica officinalis, Ocimum tenuiflorum and Withania somnifera*. *Emblica officinalis* Geart. (Amla) belonging to family Euphorbaceae, is one of the widely used medicinal herb in Ayurveda. Phyllemblin / ethyl gallate, an important constituent of *E. officinalis* potentiates the action of adrenaline that helps during stress. *E. officinalis* is a good source of vitamin C consisting 700 mg in 100 g of fruit pulp [5] and has demonstrated antistress activity against various stresses like physical stress, environmental stress [6], noxious chemicals/toxins induced oxidative stress [7]. Hydrolysable tannins – embelicannin A & B, punigluconin, peduculagin, geraniin, gallic acid, ellagic acid, tannic acid are responsible for its protective activity against oxidative stress, flavonoids like rutin, quercetin and embelin are for anti-inflammatory, anti-ageing, antioxidant and estrogenic activity and alkaloids like phyllantine and phyllantidine. *E. officinalis* inhibits apoptosis and DNA fragmentation [8,9] and also reported to act as vitalizer, anti-fatigue and antihypoxia strengthening muscle tone [10].

Ocimum tenuiflorum (Synonym: *Ocimum sanctum*), commonly called tulsi, of Lamiaceae family is reputed as a holy herb because of its extensive medicinal properties. Phenolic compounds – Eugenol, methyl chavicol, essential oils - linalool, carvacrol, beta sitosterol, beta carotene, ascorbic acid, oleic acid and tannins are major phytoactives found in *O. tenuiflorum* contributes to various activities like adaptogenic/antistress, anti-inflammatory, anti-oxidant, anti-ageing, antimicrobial etc. [11] *Withania somnifera*, well known as ashwagandha of Solanaceae family, is one such herb like aforesaid herbs known for its adaptogenic/antistress activity. Withanolides, withaferin, quercetin, rutin, choline, sitosterol, stigmasterol, sitoinosides, linoleic acid, cysteine, acylsterylglucosides are the major phytoactives attributed for pharmacological actions like growth promoter, anxiolytic, antistress, antidepressant, immunomodulator, anti-inflammatory, antimicrobial, and antioxidant [12,13]. Interventions of these herbs proved to help in defending various stress like cold stress, physical/environmental stress, psychological stress, toxicant stress, metabolic stress etc. [14-16]. In perspective of the multi pharmacological activity of the aforementioned herbs, a novel formulation out of combination of these herbs was hypothesized to demonstrate adaptogenic/antistress activity. Thus, this study was conducted to evince the adaptogenic/antistress activity of the formulation to manage chronic variable stress (CVS) in rats. The mechanism by which PHF defend the severity of stress was studied in *in vitro* test systems.

II. MATERIALS AND METHODS

A. In Vivo Study

The test substance PHF used in this study is Phytocee™ and is the proprietary supplement produced by M/s Natural Remedies Private Limited, Bengaluru, Karnataka, India. It contains fruits of *Emblica officinalis*, the whole plant of *Ocimum tenuiflorum* and stem of *Withania somnifera*.

➢ *Animals and Experimental Design*

Animals used for the study were in inbred at Central animal facility, R&D center, Natural Remedies Pvt. Ltd., Karnataka, India. All animal experiments were approved (IAEC/PCL/03/06.15; IAEC PCL/06/08.09) and performed in accordance to institutional animal ethical committee of Natural Remedies Pvt. Ltd. under the supervision of Committee for the purpose of control and supervision of experiments on animals, India. Before initiation of experiments, the animals were acclimatized to laboratory environment atleast for 7 days. The animals were housed in group of three in polypropylene cage under laboratory condition with 12 h:12 h light and dark cycle at 23 ± 2 °C with 30–70% relative humidity. The animals were provided with regular laboratory animal feed (M/s VRK nutritional solutions, Pune, India) and purified UV treated water ad libitum.

➢ *Chronic Variable Stress*

Wistar rats of either sex (n=30), aged 8-10 weeks were grouped into five containing six animals in each: G1 – Normal control (0.5 % CMC), G2 – Stress control (0.5 % CMC), G3 – PHF 5 mg/kg, G4 – PHF 10 mg/kg, G5 – PHF 15 mg/kg. All groups except G1 were subjected to chronic variable stress (CVS) regimen for 16 days as mentioned in Table 1. Concurrently, animals were administered orally the respective treatment daily for 16 days. During the period of 16 days, for every four days (day 4, 8, 12 and 16), animals of all groups were subjected to forced swim test (FST) and the immobility time during the test was recorded. As a training session, rats were made to swim in a cylinder containing water up to 25 cm height, maintained at 25 ± 1 °C for 15 min (training session) and were removed, dried and returned to cages. For test session, on day 4, 8, 12, and 16, rats were made to swim for 5 min and immobility time was recorded. The immobility time is defined as the total time rat remains immobile, floating in an upright position with its head above the water [17,18]. On day 16, immediately after FST, blood was collected and serum was separated for the estimation of serum cortisol using Cortisol Homogenous Time-Resolved Fluorescence HRTF® Cortisol assay (Cisbio, USA).

Day	Stressor
1, 5, 9, 13	5-min cold water swim (4 - 8 °C) Overnight fasting
2, 6, 10, 14	4 hour of wet bedding 1 min. tail pinch
3, 7, 11, 15	2 h immobilization Lights on overnight
4, 8, 12, 16	Forced swim test

Table 1:- Chronic Variable Stress Pattern

➢ *Swim Endurance Test*

Either sex of Swiss albino mice (n=18) aged 8-10 weeks were grouped into three containing six animals in each: G1 – Normal control (Vehicle: 0.5 % CMC) *p.o*, G2 – PHF 35 mg/kg *p.o*, G3 – PHF 100 mg/kg *p.o*. All animals were treated for 10 days. On day 10, one hour after treatment, animals were subjected to swim endurance test. The animals were allowed to swim in a tank filled with water (30 cm height and 70 cm diameter containing water up to 19 cm height, maintained at 25 ± 1 °C). The time at which animal drown into the water was recorded and considered to be the measurement of adaptogenic activity [19].

B. In Vitro Studies

➢ *Test Substance and Reagents*

Extract of PHF was used for *in vitro* assays. GeneBLAzer® CRHR1 CHO-K1 DA kit, Corticotropin releasing factor (CRF), Dulbecco's Modified Eagle Medium (DMEM), LiveBLAzer–FRET B/G (Invitrogen, USA), Fetal bovine serum (FBS), Forskolin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), dimethylsulfoxide (DMSO), Antalarmin (Sigma), Cortisol ELISA kit (Cayman chemical, USA), SYBR green

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master mix (BioRad, USA), Nu-serum, ITS+ premix, Verso cDNA synthesis kit, Oligo deoxythymine (dT) primer (ThermoFischer Scientific, USA) were utilized for *in vitro* experiments were used for *in vitro* assays.

➢ *Cells Culture Conditions*

Human adrenocarcinoma (H295R) (ATCC® CRL-2128™) cell lines were obtained from American Type Culture Collection. Human Corticotropin Releasing Hormone Receptor 1 (CRHR1) expressed CHO-K1 DA (Division Arrested) cells (GeneBLAzer®) were procured from Invitrogen (USA). It contains a beta-lactamase (bla) reporter gene. These CRHR1 CHO-K1 cells were validated for EC_{50} concentration of corticotropin releasing factor (CRF) and Z'-factor. Cells were cultured and maintained at 37 °C under 5% CO_2 humidified air.

➢ *Cytotoxicity Assay*

Cytotoxicity of PHF on human adrenocarcinoma (H295R) cell and Chinese hamster ovary (CHO-K1) cells was studied using MTT assay. Briefly, 100 µl of medium containing H295R cells and CHO-K1 cells were added separately to each well of 96 well microtiter plate (10,000-50,000 cells/well). After 24 h, the formed monolayer was washed and 100 µl of PHF was added to H295R and CHO-K1 cells at concentration ranging from 5 – 160 µg/ml and 0.16–100 µg/mL respectively. Cells were incubated for 24 hour followed by incubation with 100 µl MTT for 4 hours at 37°C, 5% CO_2. 100 µl of DMSO was then added to solubilize the formed formazan and the absorbance was read at 570 nm to calculate cell viability.

➢ *CRHR1 Receptor Assay*

CHO-K1 cells (10000 cells/well) were added to 384 well plate and incubated for 20 h at 37 °C and 5% CO_2. The cells were treated with PHF at noncytotoxic concentration, 0.16 to 100 µg/mL (data not shown) or 50µM Antalarmin hydrochloride, an antagonist for 30 min, followed by incubation for 4 h with 50 pM of corticotropin releasing factor (CRF) (agonist). 8 µL of the LiveBLAzer–FRET B/G substrate mixture was then added and β-lactamase expression was estimated to quantify CRHR1 activation using BMG FLUOstar Optima (BMG Labtech, Germany) by detecting coumarin fluorescence at 410 nm excitation/460 nm emission spectra, and coumarin-fluorescein complex at 410 nm excitation/520-nm emission spectra [20].

➢ *Cortisol Release Assay*

H295R cells were grown in DMEM, supplemented with 2.5% Nu-serum, ITS+ premix, at 37 °C, 5% CO_2 until confluent. The cells were dissociated and appropriate cell density was prepared and incubated for 24 hrs at 37°C with 5% CO_2. The monolayer was trypsinized and the cell count was adjusted to 5 X 10^5 cells/ml. To the each well of 96 well microtiter plate, 100 µl (50,000/well) of H295R cell suspension was added. After 24 h, the cells were incubated with PHF at various concentrations (5 – 40 µg/ml) and 10 µM forskolin at 37° C in 5% CO_2 for 24 h [21,22]. Post incubation, the cell supernatant was analyzed for cortisol levels by Cortisol ELISA kit according to kit instruction (Cayman chemical, USA).

➢ *11β-hydroxysteroid Dehydrogenase Type 1 Gene Expression*

The trypsinized monolayer of H295R cells were seeded to 6 well plates at a density of 1 X 10^6 cells under regular growth conditions. The cells were incubated with PHF at various concentrations, 20 – 40 µg/ml along with 10 µM forskolin for 24 h at 37° C with 5% CO_2. Total RNA was extracted from treated cells using RNA isolation reagent (TRIzol method). 2 µg of total RNA was used to synthesize cDNA using Verso cDNA synthesis kit with oligodeoxythymine (dT) primer. The PCR amplification was carried out in a reaction volume of 10µl containing 2 µl of cDNA and 5 µl of SYBR Green master mix. The Real Time PCR reaction (in CFX96 Real Time system, Bio-Rad, USA) was carried out using primers given in Table 2. The RNA expression levels were normalized to that of housekeeping (Glyceraldehyde 3-phosphate dehydrogenase) gene [23].

C. Statistical Analysis

Statistical analysis was performed in SPSS version 21 (IBM, USA) or GraphPad prism version 5.01 (GraphPad software, Inc., USA). Raw data were analyzed using oneway ANOVA followed by *post–hoc* test. Statistical significance was set at $p \leq 0.05$.

Gene	Sequence
GAPDH	Forward primer - ACATCATCCCTGCCTCTAC Reverse primer - GGCAGGTTTTTCTAGACGG
11β-HSD1	Forward primer - CCAATCCATCCCAGGGCATT Reverse primer - TTCATCACGTCCTTGGGCTC

Table 2:- Sequence of Primers for 11 beta-HSD1 and GAPDH

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase; HSD - Hydroxysteroid dehydrogenase

III. RESULT

A. Effect of PHF on Chronic Variable Stress

Rats were measured for their immobility time during FST to evaluate their coping ability at the time of stress. Immobility time was significantly high in stressed control group when compared to normal control. Whereas concurrent treatment with PHF at all dose levels (5-15 mg/kg) significantly decreased immobility duration right from day 4 to day 16 when compared to stress control animals (Fig. 1). Cortisol, a stress hormone was found to be significantly increased in stressed control group when compared to normal control group. While, serum cortisol level found to be decreased significantly in the group treated with PHF at a dose of 15 mg/kg (Table 3).

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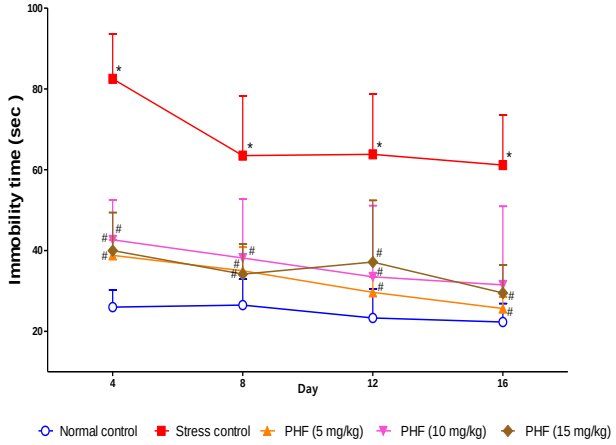
Groups	Serum cortisol (ng/mL)
Normal control	3.38 ± 0.63
Stress control	4.74 ± 0.75*
PHF (5 mg/kg)	4.80 ± 0.93
PHF (10 mg/kg)	4.05 ± 0.99
PHF (15 mg/kg)	3.67 ± 0.72#

Table 3:- Effect of PHF on serum cortisol levels in rats subjected to CVS

n=5; Data are presented as mean ± SD; *p < 0.05, Stress control Vs Normal control; # p < 0.05, Treated groups Vs Stress control; PHF- Polyherbal Formulation



n=6; Data are presented as mean ± SD; *p < 0.05, Stress control Vs Normal control; # p < 0.05, Treated groups Vs Stress control; PHF- Polyherbal formulation

Fig 1:- Effect of PHF on Immobility Time during FST in CVS

B. Effect of PHF on Adaptogenic Activity

The adaptogenic activity of PHF was determined by swim endurance test. Treatment with PHF at 35 and 100 mg/kg for 10 days increased swimming time by 1.73 and 1.52 fold respectively than compared to normal control group (Table 4).

Groups	Swim time (min)
Normal control	122.33 ± 9.97
PHF (35 mg/kg)	211.17 ± 23.76*
PHF (100 mg/kg)	185.83 ± 37.26

Table 4:- Effect of PHF on Swimming Time in Stressed Mice

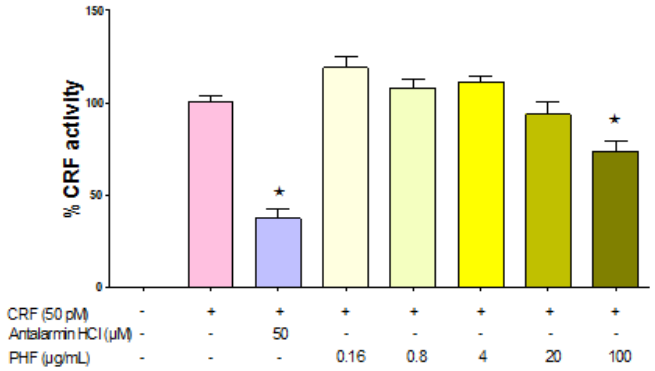
n=6, Data are presented as mean ± SD; *p < 0.05, Treated groups Vs Vehicle control; PHF- Polyherbal formulation

C. Effect of PHF on CRHR1 receptor

PHF did not affect the viability of CHO-K1 DA cells at tested concentrations (0.16 - 100 µg/mL) (data not shown). PHF at noncytotoxic concentration, 100 µg/mL, demonstrated significant antagonistic activity on CRHR1. PHF at 100 µg/mL exhibited antagonism of 26% on CRF induced CRHR1 activity. While Antalarmin hydrochloride at 10 µM produced 63 % antagonistic effect on CRHR1 (Fig. 2).

D. Effect of PHF on Cortisol Release

PHF at concentrations of 5 - 160 µg/mL did not show any cytotoxicity on H295R cells (data not shown). PHF (of 5 - 40 µg/mL) decreased cortisol release in concentration-dependent manner. PHF at 40 µg/mL showed highest inhibition of 90.31 % in forskolin induced cortisol release. PHF, even at lower concentration, 5 µg/ mL,showed ~40% inhibition of cortisol release (Table 5).



n=3, Data are presented as mean ± SD; *p < 0.05, Treatment Vs CRF stimulated control; PHF- Polyherbal formulation, CRF - Corticotropin releasing factor

Fig 2:- Effect of PHF on CRF Induced CRHR1 Activity in CHO-K1 DA Cells

E. Effect of PHF on 11β-hydroxysteroid dehydrogenase type 1 gene expression

PHF downregulated 11β-hydroxysteroid dehydrogenase type 1 (*11β- HSD1*) expression at tested concentrations (20 and 40 µg/mL). At a concentration of 20 µg/ml and 40 µg/ml of PHF, downregulation of 7.4 fold and 11.5 fold was observed respectively when compared to forskolin (10 µM) (Fig. 3).

Treatment	Cortisol concentration (pg/ml)	Percent Inhibition (%)
Control	1161.80	-
Forskolin (FSK) 10 µM	2308.03	-
FSK + PHF 5 µg/ml	1430.39	38.03
FSK + PHF 10 µg/ml	1205.92	47.75
FSK + PHF 20 µg/ml	477.92	79.29
FSK + PHF 40 µg/ml	223.70	90.31

Table 5:- Effect of PHF on Cortisol Release in H295R Cells

n=3; FSK- Forskolin, PHF- Polyherbal formulation

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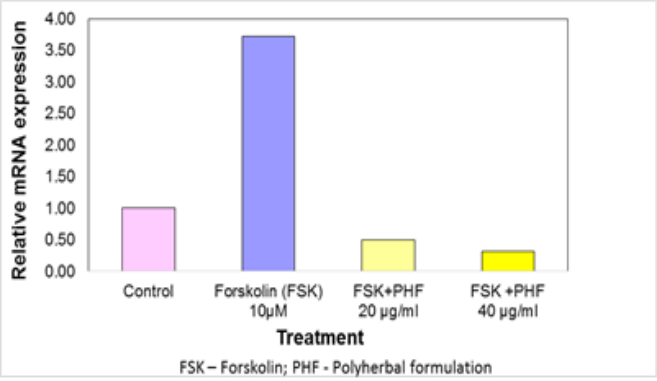

Fig 3:- Effect of PHF on mRNA Expression of *11β-HSD1* in H295R

IV. DISCUSSION

Emblica officinalis, Ocimum tenuiflorum and Withania somnifera were well known individually for their adaptogenic and multipharmacological activity. However, the activity of PHF against chronic variable stress that closely mimics the realistic stress condition of humans and animals has not been studied. Hence the present study was focused to evaluate the efficacy of PHF against variable stress and to delineate their plausible defending mechanism during stress.

Adaptation to stress is natural and self-manageable unless the events overrule the limit and become pathological. Variability in stressor is a crucial factor that is associated with behavioural adaptations to stress [24]. Chronic variable stress is highly effective than chronic repeated stress and is the most implemented model to study adaptogenicity [25]. Hence in the present study, chronic variable stress (CVS) paradigm was employed which has a design of sequence of various stressors for 16 days [17]. Immobility time during FST is directly related to the ability of animals to manage/adapt stressful condition [26]. Animals under stress loses its threshold to cope the FST and results in longer immobility time [27]. An intervention that decrease the immobility time is considered to be adaptogenic/antistress agent [28]. As aforesaid, animals treated with PHF reduced the immobility time during FST signifying its adaptogenic/antistress activity. Also, PHF increased swimming time by 1.7 fold than the normal group that reflects increased adaptability/energy and vitality effects of treatment the treatment. PHF found to corroborate the adaptogen criteria by acting as an energy enhancer and anti-fatigue [29].

Stress activates HPA-axis that release cortisol, a stress hormone by adrenal, plays a major role in body's fight-flight response to stress. However, sustained increase in cortisol level during chronic stress seriously impacts the physiological function altering the immune function and triggers the cell damage. Candidate to be called the antistress agent were likely to decrease serum cortisol level. Decreased serum cortisol level in PHF treated group of animals in comparison to untreated stressed group demonstrated it to an antistress agent. Effect of PHF against chronic variable stress proves PHF to be potent adaptogen that could defend stressful condition irrespective of the stimulus variability [24].

Chronic stress reputed to be managed by various mechanisms on hypothalamic pituitary adrenal (HPA) axis, autonomic nervous system, extra-adrenal pathways involving neuropeptides and neurotransmitters [30]. In the present study, the possible mechanism on HPA axis mediated by Corticotropin-releasing hormone receptors (CRHR) was investigated. Corticotrophin-releasing hormone (CRH) is a key regulator in HPA axis that is triggered initially during fight or flight response. CRH regulates humoral response to stress by activating adrenocorticotropin (ACTH) through CRHR, a G protein-coupled receptor and releases cortisol that accounts for stress-related behavioural changes. In chronic stress, CRH, upon eventual activations release glucocorticoids (cortisol) resulting in stress-related disorders. CRH acts on CRHRs among which CRHR1 is the key receptor. Hence, antagonistic effect on CRHR1 proved to be the therapeutic target for stress management that control cortisol release [31-33]. PHF found to act an antagonist for CRH against CRHR1 expressed CHO-K1 cells. The study demonstrated that PHF is CRHR1 antagonist, impeding ACTH-mediated effects. Cortisol that accounts for a stress induced behavioural and pathological changes is a salient biomarker of HPA axis activation in chronic stress. Intervention that inhibits cortisol release in chronic stress could prevent major stress-related pathological and behavioural changes. Forskolin was found to induce cortisol release in H295R cells. Forskolin increases cortisol by adenylyl cyclase C mediated cAMP release which is important for hormone synthesis [34]. In that way, PHF treatment showed significant inhibition of forskolin induced cortisol in H295R cells. This demonstrates that PHF could modulated cAMP, a second messenger pathway and thus the feedback control of cortisol release. This study corroborates with *in vivo* cortisol release in stressed rats that demonstrated evident inhibition of cortisol release.

11β-hydroxysteroid dehydrogenase type 1 (*11β-HSD1*), is a bidirectional enzyme that participate in glucocorticoid metabolism. It is hormone regulator at tissue level, interconverts inactive cortisone to active cortisol. Inhibition or downregulation of *11β-HSD1* in HPA-axis found to possess a significant therapeutic role in treating stress and related metabolic/pathological disease that includes insulin resistance, obesity, structural deterioration and neuroendocrine dysfunction [35]. PHF at tested concentration downregulated *11β- HSD1* expression to the maximum of 7.4 fold than forskolin induced expression. This investigation demonstrate that PHF could be employed for both metabolic and glucocorticoid-associated CNS disorders. The effect of PHF, Phytocee™ could be attributed to the combined effect of its individual ingredient namely *Emblica officinalis, Ocimum tenuiflorum* and *Withania somnifera* which are rich in polyphenols, withanolides and triterpenoids. These phytoactives are earlier proved to be responsible for antistress/adaptogenic effect [36-39].

Thus possibly because of the aforestated mechanisms PHF could be reputed as an antistress and adaptogen as it closely satisfies the criteria defined by Brekhman as follows (EMEA): PHF is nontoxic [40], nonspecific with increased resistance to adverse factors and has a normalizing effect on system during pathological condition.

V. CONCLUSION

The study indicate that PHF (Phytocee^{TM}) could be employed as antifatigue/physical endurance enhancer and antistress against chronic variable stress. The effect of PHF could be because of its direct influence on HPA axis in reducing cortisol levels and on second messenger pathway and 11β- hydroxysteroid dehydrogenase type 1. However, a close scrutiny in target species is recommended.

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