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Stress, Adaptogens and Their Evaluation: An Overview

Arunabha Ray*, Kavita Gulati and Rashmi Anand

Department of Pharmacology, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi-110007, India

Abstract

Stress is any physical, psychological and/or environmental stimulus capable of altering physiological homeostasis, and the ability to cope with such stressful stimuli is a crucial determinant of health and disease. Stress itself is a defense response of an organism to external factors activating several protective mediators but prolonged stress can be detrimental and have deleterious effects on the biological system. Stress in its different forms is capable of precipitating a variety of pathophysiological states and almost all organs/systems have been implicated. Conventional therapies though effective have a plethora of adverse effects and hence there is a dire need for developing better therapeutic options/strategies. The term adaptogen is used primarily to describe agents from herbal sources, which increase the resistance to aversive inputs and result in overall protective effects during stress. In recent years, adaptogens have gained considerable attention because of their safety, cost effectiveness and efficacy in stress modulating effects which could improve the quality of life in both diseased and healthy persons. This review will attempt to summarize and critically analyze the recent concepts in the area of such adaptogens and their evaluation methodologies.

Keywords: Stress; Adaptogens; *Withania somnifera*; *Panax ginseng*; *Ocimum sanctum*; *Azadirachta indica*; Evaluation methods

Introduction

Stress

All organisms are in a state of dynamic equilibrium or homeostasis which is constantly challenged by internal/external aversive stimuli called stressors. Stress occur when such homeostasis is threatened and a set of physiological and behavioral adaptive responses come into play. These coordinated adaptive responses enhance the probability of survival. Such stress responses are mediated by the stress system and is orchestrated by the brain (CNS) in conjunction with peripheral organs. Failure to adapt to stressors results in dysregulation of the stress system and may lead to psychiatric, endocrine/metabolic and/or autoimmune diseases or increase vulnerability to such diseases [1]. Stress and stress related disorders account for over 70% of the global illnesses. Studies on the pathophysiological aspects of stress has been a subject of research for long and Cannon in 1929 [2] first suggested that any aversive emotional stimulus is capable of causing physical damage to the body and can produce disease states like anxiety and depression, hypertension, immunosuppression, endocrine disorders, diabetes mellitus, peptic ulcer, etc. In 1936, Selye [3] introduced the concept of stress in biology and medicine and defined it as a non-specific response of the body to any environmental demand placed upon it. He described a response triad viz. adrenal hypertrophy, gastrointestinal ulceration, and thymolymphatic involution/atrophy, which should be elicited by any stressor and suggested the HPA axis as the principal regulator of the stress response. He considered the adrenal cortex to be the organ of integration during stress. Selye also introduced the term general adaptation syndrome (GAS) with its three successive phases: the alarm reaction, stage of resistance, and exhaustion stage [3]. First, an initial alarm reaction, analogous to Cannon's 'fight or flight' response, second, a stage of adaptation associated with resistance to the stressor, and eventually a stage of exhaustion and organism's death. However, his idea of non-specificity of stress response was replaced by one of specificity and accordingly the concept of stress was altered. Stress and stress research has evolved considerably over the years and it is now increasingly being recognized as a highly interactive phenomenon and terms like stress syndrome, stress system and allostasis have emerged. Allostasis is the process of adaptation of the body upon the exposure to various stressors, wherein mediators like cortisol or adrenaline are released which in turn promote adaptation. However, when these stress mediators are not turned on adequately during stress, or when they are not shut down after the stressor, or when there is overuse, it leads to "allostatic overload". It is thus implied that the outcome of long-term physical or mental stress would depend on adequate regulation of such allostatic load [4]. It is now well known that the CNS is central to stress and its neural networks are also vulnerable to emotional and environmental stressors which could act as trigger and orchestrate the genesis of many neuropsychiatric/neurodegenerative, cardiovascular, gastrointestinal and immunological disorders. Complex neurochemical pathways/interactions have been proposed to explain stress responses and resultant pathophysiological states and brain amines, amino acids and neuropeptides have been implicated. The activation of the hypothalamic-pituitary-adrenocortical axis and renin-angiotensin system plays a major role in the regulation of stress response. Neuroendocrine components activated by stressors include the increased secretion of epinephrine and norepinephrine from the sympathetic nervous system and adrenal medulla. Further, the release of corticotropin-releasing factor (CRF) induces the secretion of pituitary adrenocorticotropin (ACTH), leading to secretion of glucocorticoids by the adrenal gland. CRF coordinates the endocrine, autonomic, behavioral and immune responses to stress and also acts as a neurotransmitter or neuromodulator in the amygdala, dorsal raphe nucleus, hippocampus and locus coeruleus, to integrate brain multi-system responses to stress. The response of the cardiovascular system to stress has been ascribed mainly to excessive stimulation of catecholamine and involves increased cardiac output and vascular resistance, lipid mobilization, and stimulation of platelet aggregation

***Corresponding author:** Arunabha Ray, Department of Pharmacology, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi-110007, India, Tel: 91-11-27402403, +919818037595; Fax: 91-11-27666549; E-mail: arunabha14@yahoo.co.in

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[5]. When exaggerated, these effects can cause hypertension, atherosclerosis and ischemic heart disease. The gastrointestinal and immune systems are particularly susceptible to stressful inputs and results in complex disease states. More recently, it has been proposed that the CNS and its neural circuitry could regulate peripheral stress effects on the gastrointestinal and immune systems and concepts like the CNS-gut and CNS-Immune axis have emerged [6].

The CNS is highly susceptible to the detrimental effects of reactive oxygen species (ROS) due to their high metabolic rate, rich composition of fatty acids prone to peroxidation, high intracellular concentrations of transition metals capable of catalyzing the formation of reactive hydroxyl radicals, low levels of antioxidants, and reduced capability to regenerate. Generated free radicals can attack proteins, polysaccharides, lipid bilayers, and DNA, causing cellular oxidative damage. Damage of nervous tissue by free radicals may contribute to neuronal loss in cerebral ischemia and may also be involved in normal aging and neurodegenerative diseases, e.g., epilepsy, schizophrenia, Parkinson's, Alzheimer's, and other diseases [7]. More recently, it has been suggested that the gassotransmitter, nitric oxide (NO) and its complex signaling pathways could influence stress effects in a concentration dependent manner [8].

Classically anti-stress agents are those which help to combat or cope with stressful situations and include both typical and atypical anxiolytic agents like benzodiazepines, beta blockers (propranolol), and 5-HT1A partial agonist (Buspirone), but their pharmacodynamics and pharmacokinetic profiles are different. Benzodiazepines facilitate GABA-ergic neurotransmission and also have sedative/hypnotic, anticonvulsant and muscle relaxant effects. Further, long term treatment with benzodiazepines, incurs the risk of cognitive dysfunction, dependence and induces withdrawal signs upon discontinuation. Similar related problems are also associated with the other conventionally used agents for stress related conditions [9-11]. Although, modern medicine has developed several drugs to cope with stress, but still the results are unsatisfactory and the prevention and management of stress and stress related disorders still remains a problem. Hence, balancing safety and efficacy as well as identification of the right target is essential for developing drugs in stress and stress related disorders. In addition there is a dire need to develop a battery of tests, which need to be comprehensive and confirmatory to screen new agents with anti-stress profile.

Adaptogens

Herbal drugs play an important role in the traditional systems of medicine (*viz.* Ayurveda, Unani) and have been used in medical practices since antiquity. Herbal products contain phytochemicals which may have the potential to act as preventive or therapeutic agents against various human diseases. Life style diseases which are commonly precipitated by stress are an area where they could be of great benefit. Historically, the concept of adaptogens dates back thousands of years to ancient India and China, but in the modern era the earliest studies did not begin until mid-part of the twentieth century. In 1947, Dr. Nicholas Lazarev, a Russian scientist, while looking for substances that could improve general resistance to toxins and correct the general adaptation reaction to all kinds of stressors to help soldiers overcome fatigue and improve their performance on the battlefield, started giving stimulants like amphetamines to combat long term stress. The hypothesis was that stimulants increase the sympathetic nervous system activity, produce euphoria, increase alertness and ability to concentrate on mental tasks. However, he soon realized that the stimulants can impair mental function and lead to psychotic symptoms, disturb the sleep structure and produce drug withdrawal symptoms so he switched his focus to natural source alternatives. He defined them as adaptogens that allow the body to counter adverse physical, chemical, or biological stressors by raising nonspecific resistance toward such stress, thus allowing the organism to "adapt" to the stressful circumstances. The word "adaptogen" is derived from the Greek word "adapto," which means "to adjust." Classically, adaptogens are medicinal plant products that increase the body's resistance to stressors such as trauma, anxiety and bodily fatigue. In the past they have been called rejuvenating herbs, restoratives or Rasayanas (in Ayurveda). In 1968, Breckham and Dardymov [12] formally stated that adaptogens are non-toxic substances which non-specifically increase the power of resistance against multiple stressors including physical, chemical and biological agents, and have a normalizing influence on physiology, irrespective of the direction of the change from physiological norms caused by the stressor. Under this definition, adaptogens would be nontoxic in normal doses, produce a general defensive response against stress, and have a normalizing influence on the body [10]. Adaptogens reduce the intensity and negative impact of the stress caused by mental tension, emotional difficulties, poor lifestyle habits, disease and infection, pollution and other factors Adaptogens attenuate anxiogenic responses, influence CNS and immune systems/functions, and hence may be of value in life style disorders. Ideally, they should be innocuous and cause minimal disorders in the physiological functions of an organism and have a normalizing action irrespective of the direction of the pathological state. They increase the body's non-specific resistance to internal and external stimuli and bring the dysfunctioning body's system back to normal function. These are agents which reverse/prevent stress effects and are important therapeutic moieties with complex mechanisms. Adaptogens can successfully combat the negative effects of stress and enhance body's performance, by metabolic regulators such as HPA-axis hormones, biogenic amines, neuropeptides, cytokines, nitric oxide (NO) etc. This explains why adaptogens also have anti-inflammatory, antioxidant, anxiolytic, antidepressant and amphoteric effects as well [11]. It is known that long term training results in increased blood levels of corticosterone and that a trained organism responds insignificantly to stress stimuli as compared to an organism that is experiencing the stress exposure for the first time. Reports have suggested that single dose administration of adaptogen activates corticosteroid formation however repeated adaptogen administration normalizes the corticosterone levels. Adaptogens increase the ability of the stress system to respond to stressful stimuli by moderately modulating the levels of corticosterone, biosynthesis of eicosanoids including prostaglandins E2 and F2, 5-hydroxyeicosatetraenoic acid (5-HETE), 12-HETE and leukotriene B4, arachidonic acids [12]. Adaptogens can be divided into 3 groups in terms of active ingredients a) phenolic compounds such as phenyl propanoids, phenylethane derivatives and lignans which structurally resemble to catecholamines and are found in roots and rhizomes of plants e.g. E. senticosus, R. roseaand S. chinensis fruits; b) Tetracyclic triterpenes such as cucurbitacin R diglucoside which structurally resemble corticosteroids and found in extracts of *B. alba* and *W. somnifera*; c) Oxylipins- unsaturated trihydroxy or epoxy fatty acids which structurally resemble leukotriens and lipoxines found in *B. alba* and *G. glabra* [13]. Since the introduction of adaptogens, a large variety of medicinal plants (which were used as tonics) have been studied for their adaptogenic and rejuvenating properties, to promote health and maintain resistance against infections by re-establishing body equilibrium. Adaptogens are important compounds in many ayurvedic and unani medicines for their adaptogenic, anti-oxidant and rejuvenating properties. However, apart from these properties, in present times they are known for their anti-cancer, hepatoprotective

and immunomodulatory properties. Some important medicinal plants with adaptogenic properties are listed in Table 1.

A brief account of some important adaptogens is given below:

***Withania somnifera*:** It is commonly known as Ashwagandha (Indian ginseng or winter cherry) and the name is due to peculiar odor of this herb which resembles to that of a sweaty horse. It is cultivated in Madhya Pradesh, Punjab, Gujarat and Rajasthan. Nagauri is a local variant and its roots have more starch content as compared to other varieties. Poshita and Rakshita are the two high yielding varieties (HYV) released from CSIR and CIMAP (Lucknow). In Ayurveda, *Withania somnifera* (WS) is classified as a Rasayana- a group of plant-derived drugs reputed to promote physical and mental health, augment resistance of the body against disease and adverse environmental situations, revitalise the body in debilitated conditions and increase longevity. Over 35 chemical constituents have been isolated: alkaloids like isopelletierine, anaferine, ashwagandhin, cuscohygrine, anahygrine, topine, saponins, sitoindosides, withanolides, withaferin A, withasomnidieone, withanone etc. Ashwagandholine of WS roots produced a taming and a mild depressant (tranquilizer) effect on the central nervous system in monkeys, cats, dogs, albino rats, and mice [14]. Active glycowithanolides of WS (10 or 20 mg/kg i.p) given once daily for 21 days to groups of six rats showed dose-related increase in all enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) [15]. This implies that WS does have an anti-oxidant effect in the brain. The antioxidant effect of its root extract (100 mg/kg) on stress-induced lipid peroxidation (LPO) in mice and rabbits have been shown and LPO blood levels were increased by i.v. administration of 0.2 mg/ kg of lipopolysaccharides (LPS) from Klebsiella pneumonia and 100 mg/kg of peptidoglycans from *Staphylococcus aureus* [16]. Immunomodulatory effects of WS root extract in three myelosuppression models in mice: cyclophosphamide, azathioprin and prednisolone have been shown [17]. As compared to untreated controls, WS treated mice showed significant increases in hemoglobin concentration, red blood cell count, white blood cell count, platelet count, and body weight. They have also reported significant increases in haemolytic antibody responses toward human erythrocytes which indicated immuno-stimulatory activity. In another study, mice given WS root extract 1.4 g/kg by gavage, daily for 20 days significantly increased the serum levels of triiodothyronine (T3) and tetraiodothyronine (T4), while the hepatic concentrations of glucose 6-phosphatase activity and hepatic iodothyronine 5'-monodeiodinase activity did not change significantly. In addition, it significantly reduced hepatic lipid peroxidation and increased the activity of SOD and catalase -thus suggesting that WS stimulates thyroid activity and also promotes hepatic antioxidant activity [18]. In a clinical trial, it has been reported that, WS powder given 3 g/day for one year to 101normal healthy male volunteers aged 50-59 years showed significant increase in hemoglobin and RBC count, and improved hair melanin [19]. They also reported that there was improvement in sexual performance in 71 percent of the subjects. Anti-stress activity for this plant has been shown as powdered roots of WS suspended in 2% acacia (100 mg/kg in 1ml orally) when administered to mice daily for 7 days; and on 8th day swimming endurance test was given a significant anti-stress activity was found [20]. Growth inhibitory effects of WS in Sarcoma 180 (S-180), a transplantable mouse tumor have been reported [21]. Ethanolic extract of WS root (400 mg/kg and 1000mg/kg, daily for 15days) after intra-dermal inoculation of 5×10^5cells of S-180 in BALB/c mice resulted in complete regression of tumor. A 55% regression was obtained at 1000 mg/kg; although, it was a lethal dose in some cases. WS was also found to act as a radio- and heat sensitizer in mouse S-180 and in Ehrlich ascites carcinoma. Antitumor and radio sensitizing effects of withaferin A (a steroidal lactone) have been shown in mouse Ehrlich ascites carcinoma *in vivo* [22]. Withaferin A gave a radio sensitizer ratio of 1:5 for in vitro cell killing of V79 Chinese hamster cell at a non-toxic concentration of about 2 mM/L. These studies are suggestive of antitumor activity as well as radio and heat enhancement effects of WS. In a clinical study, the possible use of WS for arthritis has been reported in a double-blind, placebo-controlled cross-over study [23]. 42 patients with osteoarthritis were randomized to receive a formula containing WS or placebo for 03 months. During the pretreatment and treatment phase, pain and disability scores were evaluated weekly while ESR and radiological studies were conducted monthly. WS significantly reduced the severity of pain and disability scores, although no significant changes in radiological appearance or ESR were noted. In another study, methanolic extract of WS (10 mg/kg) was given in cotton-pellet implanted rats for 4 days and the pellets were harvested on day 4. The results showed that WS extract inhibited the granuloma formation in cotton-pellet implantation in rats and the effect was comparable to hydrocortisone sodium succinate (5 mg/kg) treatment [24]. It has been reported that WS reversed the cold swimming-induced increases in plasma corticosterone, phagocytic index, and avidity index to near to control levels - thus indicated its anti-stress effects [25]. Neuroprotective role of WS against glutamate induced toxicity in the retinoic acid differentiated rat glioma (C6) and human neuroblastoma (IMR-32) cells has also been reported [26].

Name of plant	Common name	Part of plant used
Withania somnifera	Ashwagandha (Indian Ginseng)	Root
Panax ginseng	Ginnsuu (Asian Ginseng)	Root
Bacopa monniera	Brahmi	Leaf
Zingiber offcinale	Zingiber	Rhizome
Rhodio laimbricata	Rose root	Root
Asparagus racemosus	Shatavari	Root
Ocimum sanctum	Tulsi (Holy basil)	Leaf
Momordia charantia	Bitter Melon (Karela)	Plant
Curcuma longa	Turmeric (Haldi)	Rhizome
Azadirachta indica	Neem	Leaves
Allium sativum	Garlic	Rhizome

Table 1: List of some important medicinal plants with adaptogenic properties.

***Panax ginseng*:** *Panax ginseng* (PG, Asian ginseng) is a popular traditional Chinese medicine in which the active ingredient is the ginsenosides. Numerous studies support Asian ginseng's effectiveness at improving a person's ability to withstand stress, improve work performance and quality, and enhance mental function [27]. The herb has been reported to stimulate the hypothalamo-pituitary-adrenal (HPA) axis and increase the release of adrenocorticotropic hormone (ACTH) thus increasing adrenal hormone secretion. It also can counteract the shrinkage of the adrenal gland caused by corticosteroid drugs. In a recent in vitro study, it was found that Asian ginseng extract inhibited hydroxyl radical formation and they hypothesized that this antioxidant effect may be responsible for ginseng's wide range of pharmacological uses [28]. In a double-blind controlled clinical study, 36 noninsulin-dependent diabetic patients were treated with 100 mg or 200 mg of PG or placebo for eight weeks, and ginseng treated subjects showed elevated mood, improved physical activity and performance, improved glycosylated hemoglobin, and reduced fasting blood sugars and body weight as compared to the placebo group [29]. Referred to as a classic adaptogen, PG has been shown to increase RNA and protein content in the muscle and liver tissue of laboratory animals which may

be the reason that makes ginseng such a highly regarded tonic [30]. PG is said to tonify the lungs while strengthening the spleen and stomach and calming the spirit. Studies show this plant to be antidepressant, anti-diabetic and antihypertensive [31]. While evaluating the effect of ginseng in various forms, *viz.* cooked, dried and fresh root, in 1987 cancer patients, researchers found that the risk of developing certain cancers were less in ginseng users (for one year) as compared to that of others, and this risk continued to decrease with use up to 20 years. In particular, ginseng was found to protect against cancers of the mouth, esophagus, stomach, colorectum, liver, lung, pancreas and ovaries, and the authors inferred that ginseng had anti-cancer properties [32]. American ginseng (*Panax quinquefolius*) and Siberian ginseng (*Eleutherococcus senticosus*) are also known for their adaptogenic potential.

Ocimum sanctum: *Ocimum sanctum* (OS) is commonly known as Tulsi or Holy Basil and the leaf is the most commonly used part of the plant. It is known for its religious, spiritual sanctity and its important role in Ayurveda and Unani system for common colds, headaches, stomach disorders, inflammation, heart disease, various forms of poisoning and malaria. Its constituents are eugenol, euginal, urosolicacid, vicenin, carvacrol, linalool, limatrol, caryophyllene, methyl carvicol while the seeds are rich in fatty acids, sitosterol and leaves have mainly sugars and anthocyanins. OS has been well documented to have anti-stress effects [33]. Anti-fatigue effects of OS (300 mg/kg) in rats have been reported by observing increase in swimming time, change in body weight, lipid peroxidation, lactic acid, glycogen and parameters like heamoglobin (Hb%), Blood Urea Nitrogen (BUN) and Creatine Kinase (CK) [34]. In experimentally induced diabetes, increased glucose tolerance, insulin levels, glycogen, hemoglobin and protein levels and a consequent decrease in the blood glucose, glycosylated hemoglobin and urea were seen and have been attributed to its corticosteroid inhibition property and anti-peroxide effects [35]. OS leaf extracts also stimulate insulin secretion from perfused pancreas, isolated islets and clonal pancreatic beta-cells and have an anti-diabetic action [36]. However some researchers have demonstrated anti-hyperlipidemic and antioxidant effect of OS seed oil by a significant increase in the activity of anti-oxidant enzymes such as superoxide dismutase and catalase level in extract-treated group as compared to controls [37]. Other reports have shown that it inhibits the hypercholesterolemia-induced erythrocyte lipid peroxidation activity and protects the aortic tissue from hypercholestrolemia-induced peroxidative damage [38]. Antibacterial activity of OS against *Staphylococcus aureus*, *Bacillus pumius* and *Pseudomonas aeruginosa*, has been attributed to linoleic acid present in it [39]. Tender OS leaves boiled with tea have been reported to have anti-dengue properties [40]. Anti-convulsant activity of ethanolic extract of OS leaves was observed as it prolonged the time of lost reflex in mice in response to pentobarbital, decreased the severity of electroshock and pentylenetetrazole-induced convulsions, decreased apomorphine-induced fighting time and ambulation in 'open field' studies [41]. Radioprotective effect of OS (40 mg/kg) and amifostine (radioprotectant, 200 mg/kg) have been demonstrated on the salivary gland of rats after therapeutic radioiodine exposure [42]. Its two flavonoids: orientin and vicenin and two synthetic compounds WR-2721 and 2-mercaptopropionyl glycine (MPG) have been compared by examining chromosome aberration in cells of bone marrow in irradiated mice. WR-2721 was found to be the most effective against complex aberrations followed by vicenin while MPG was the least effective. This shows that OS flavonoids may be promising as radioprotective agents in humans. Studies have demonstrated hepatoprotective effects of the herb in lead-induced toxicity in Wistar rats [43]. Anti-cancer effects of vicenin-2, both as a single agent and in synergistic combination with docetaxel in prostate cancer, have been reported [44]. Further, it has been suggested that linolenic acid present in ocimum fixed oil has anti-inflammatory activity and its capacity to block both the cyclooxygenase and lipoxygenase pathways of arachidonate metabolism could be the mechanism for these anti-inflammatory effects.

***Azadirachta indica*:** *Azadirachta indica* (AI) is commonly known as Neem and almost every part of the tree has been used to treat different human ailments. It is regarded as a household pesticide. The extract of bark, leaves, fruits and roots has been used to treat leprosy, intestinal helminthiasis and respiratory disorders in children. The bark extract is used as tonic, astringent and useful in relieving fever, thirst, nausea, vomiting and skin diseases. Other reports on the biological and pharmacological studies showed antiviral, anti-inflammatory, immunomodulatory, antipyretic, antioxidant and adaptogenic properties. Many researchers have reported neem to have anticancer properties [45] against the DMBA and BαP induced neoplasm by enhancing the activity of MGMT (6-methylguanine-DNA methyltransferase) enzyme which detoxifies alkylguanines and thereby causing time-dependent demethylation of methylguanine and consequently prevents its cytotoxic effects. Recently, it has been reported that nimbolide and azadirachtin inhibited the development of DMBA-induced hamster buccal pouch carcinoma by preventing the activation of procarcinogen, oxidative DNA damage and upregulation of antioxidant and carcinogen detoxifying enzymes [45]. Some studies have suggested that neem leaf can be used as a substitute to granulocyte colony stimulating factor. GCSF is used to reduce side effects during cancer treatment with cisplatin and 5-FU, but it itself is expensive and also promotes angiogenesis and tumor development. AI leaf extract has been reported to cause upregulation of marker enzymes, such as alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase and the oxidative damage during skin tumor induction is inhibited. It also increased levels of the reduced glutathione. Nimbolide arrested the HT-29 (human colon carcinoma cells) in G2/M and G0/G1 stages by upregulation of p21 which is a well-known down-stream effector of the anticancer p53 gene. AI leaf extract has been reported for induction of apoptosis among the prostate cancer cells using DNA fragmentation and cell viability assays. It has been shown that treatment with neem extract suppressed bcl-2 protein expression, which is a strong pro-survival factor in cancer cells and increased the pro-apoptotic Bax protein expression. In choriocarcinoma (BeWo) cells, nimbolide, induces apoptosis via the mitochondrial pathway and mediates upregulation of Apaf1 and caspase-3 and decrease in the bcl2/bax ratio. AI leaf glycoprotein helps to generate carcino-embryonic antigen specific anti-tumor immune responses utilizing macrophage mediated antigen presentation [46].

Evaluation of adaptogens

Stress is any aversive stimulus that disrupts the physiological homeostasis and the biological system reacts to such stressors in a stressor type/duration/intensity dependent manner. The ability to cope with the stressor and maintain the homeostatic mechanisms are crucial determinants of health and disease. The CNS plays a pivotal role in conjunction with the neuroendocrine-immune axis and hence neurobehavioral, endocrinal and immunological parameters are used as markers to assess the impact of stress and its modulation by drugs and potential adaptogens. Stress also impacts the gastrointestinal and cardiovascular systems and a variety of cellular and molecular markers are affected. It has been hypothesized that 'stress begets stress' and emotional/environmental stress induced changes in oxidative stress

markers have been proposed. Cellular responses to stress are also assessed by measuring cellular chaperons *viz.* heat shock proteins (Hsp) and molecular markers in the form of DNA damage and bcl2/bax ratio is also known to be altered in specific stress situations.

The classical adaptogenic or anti-stress evaluation methods involve the effects of potential adaptogens on the CNS, endocrine system, gastrointestinal system, immune system, oxidative stress and other cellular/molecular markers [47]. In the CNS, anti-stress activity is studied by evaluation of emotional function, cognitive ability, co-ordination function, locomotor activity, temperature regulation, measurement of neurotransmitter levels in the brain tissue, etc. Endocrinal markers *viz.* CRF, ACTH, corticosterone, free arachidonic acid (AA), prostaglandin E2, leukotrienes (LTB4), NO levels, etc. can be assessed in blood, brain and other tissues as an index of anti-stress effects. Effects of stress on the gastrointestinal system can be evaluated in experimental models of gastric ulcers induced by restraint stress, drugs like aspirin, water immersion, etc. Immunomodulatory activity of adaptogens can be assessed by observing innate and adaptive immune responses *viz.* phagocytosis, CD4 cell counts, Th1/Th2 ratio, NK cells, macrophages, granulocytes etc. and other markers of humoral and cell mediated immunity including various cytokines. In addition, markers of oxidative stress, e.g. lipid [peroxidation, anti-oxidant defence (enzymatic and non-enzymatic) can also be assessed. Further, energy related biochemical correlates like ATP, creatine phosphate hexokinase, glycogen etc. Other general markers like changes in weight of thymus and adrenals, modulation of reflexes, survival against toxic chemicals and bacteria, ECG changes during different stressors, etc. can also be measured. Anabolic activity tests *viz.* increase of body weight and accelerated growth, as well as the increase of DNA, RNA and protein synthesis can also give an idea about adaptogenic potential [48]. It is thus implied that a single activity or test will not suffice for evaluating adaptogenic potentials but it is essential to perform a battery of tests.

Tests for Neurobehavioral Activity

Forced swimming test

Mice or rats are forced to swim in a restricted space from which they cannot escape and this induces a state of despair which can be reduced by anti-depressants, stimulants like amphetamine, caffeine and adaptogens. Animals divided into groups: control; stress control; drug treated stress group. For 10 days drug treatment is given. 36 h fasted animals on 10th day, after feeding and drug treatment; their rectal temperature are noted and exposed to force swimming for 20 min. Rats taken out and again their rectal temperature noted. Drop in rectal temperature is calculated. Drugs having adaptogenic properties will reverse the hypothermia in stress conditions [47].

Swim endurance test

This test is similar to the one described above *viz.*7 day drug treated mice are subjected to swimming endurance test individually inside a perplex glass (30 cm height × 20 cm diameter) containing water of 25 cm height maintained at 26 ± 10°C. The end point taken is the time when mice get exhausted and start drowning. The mean swimming time for each group is calculated. Adaptogen should increase the mean swimming time.

Post swimming motor function test

The test evaluates whether swim stress interferes with motor coordination of animals and adaptogens are capable of reversing these effects. Mice divided into groups *viz.* controls, graded doses of test drug and comparator agent. On 7th day, each animal is allowed to swim inside a perplex glass container (30 cm × 20 cm containing water upto 25 cm height) maintained at 26 ± 1°C. The end point taken is when the animals got exhausted and started drowning. Animals removed and allowed to recover and dry for about 5 min. Subsequently tested for muscle coordination on rotarod rotating at 15 rpm and the duration of stay on the rod is recorded. The apparatus consists of a horizontal wooden rod or metal rod coated with rubber with 3 cm diameter attached to a motor with the speed adjusted to 2 rotations per min. The rod is 75 cm in length and is divided into 6 sections by plastic discs, thereby allowing the simultaneous testing of 6 mice. The rod is in a height of about 50 cm above the table top in order to discourage the animals from jumping off the roller. Only those animals which have demonstrated their ability to remain on the revolving rod for at least 1 minute are used for the test. The test compounds are administered i.p. or orally. Thirty minutes after intraperitoneal or 60 min after oral administration the mice are placed for 1 min on the rotating rod. The number of animals falling from the roller during this time is counted. Adaptogens should increase the swimming time and duration of stay on the rotarod.

Treadmill test

This test is used to evaluate the influence of drugs on endurance, muscle metabolism, cardiovascular system, diet and circadian rhythm. The apparatus basically consists of a rotating running belt driven by a servo-controlled motor, which provides precise operator-defined tread speed. Separating panels divide the running surface into separate exercise lanes each suited for an individual animal. The floor grids can be used to apply and electric stimulus of variable length and intensity. The incline of the running surface can be steplessly adjusted to control the labor of the animal. Rats on a treadmill belt with increasing exercise at a speed of 10, 20 and 30 m/min, for 4 min with 2 min rest intervals. The end point taken is exhaustion despite contact with a shock at the rear of the treadmill belt. Mean arterial pressure (MAP) and heart rate (HR) recorded continuously at rest and during the three different intensities of exercise.

Elevated plus maze test

The Elevated plus maze is a well-established and widely used animal model of anxiety like behavior in which rodents are faced with a conflict situation between exploring open elevated arms and a natural tendency to hide in the enclosed arms. It consists of two open opposite arms (40 × 10 cm) and two enclosed arms, of the same measurement, with 40 cm high walls. The arms are connected in such a way that the maze has a plus sign (+) look. The entire maze is elevated 50 cm above ground and placed in a quiet dimly lit room. Experimental rats were placed individually in the center of the maze facing the closed arms and will be observed for five min. The following parameters were observed: number of open arm entries, time spent on open arm entries and closed arm entries. All four paws crossing into the arm define an entry. Subsequently, the percentage of open arm entries was calculated by dividing the open arm entries by total number of entries on both open and closed arms, while the percentage of time spent on open arm exploration was calculated by dividing the time spent on open arm by total time spent on both open and closed arms. Increased percent time or percent entries in open arms are indicative of a reduced anxiety state.

Open field test

The Open Field Apparatus is used to evaluate an emotional reactivity to a relatively unfamiliar environment and consists of a square arena 96 × 96 cm with 60 cm high walls. The walls are painted white and floor

green. The floor is divided into 16 squares by parallel and intersecting white lines. Rats were placed singly in one corner of the open field and following parameters (a) Latency, (b) Ambulation, (c) Rearing and (d) Fecal pellets were observed during a 5 minute exposure period.

Hole-board test

Hole board test is a widely used method for screening the potential anxiolytic and adaptogenic character of drugs. The test is based on the assumption, that head-dipping activity of the animals is inversely proportional to their anxiety state. Changes in head-dipping behavior in the hole-board test reflects the anxiogenic and/or anxiolytic state of animal.

Hypoxia test

Hypoxia is a very severe environmental stressor as all the body functions including cellular respiration depend on oxygen supply. Any lack of oxygen adversely affects all body functions. Increase in adaptation during this stress by any drug could be considered as its major adaptogenic effect. Animals are treated with the drug for different durations. After treatment with drugs, hypoxia time is recorded by placing each animal individually in the perplex jar of 1 L capacity made air-tight with greased stoppers. The end point taken is the 1st convulsion and time for that onset is noted. An adaptogenic drug will increase the onset time of convulsion.

Tests for Endocrinal Activity

Catecholamines (adrenaline) assay

It is well documented that the catecholamine systems, especially the noradrenergic and adrenergic, both in the periphery and in the CNS, play key roles in mediating the physiological responses to stress. Also, important differences exist in the molecular mechanisms of stress responses in various catecholamine locations. This is shown by the fact that there are differences between stress-induced changes in catecholamine synthesizing enzymes in the adrenal medulla, sympatho-neural system, or different sympathetic ganglia.

Differences in catecholamine responses to acute and chronic stressors or the type of stressor is also known. Stress-triggered alterations in gene expression and the mechanisms mediating these changes is just beginning to be elucidated and such stress triggers long-term changes in gene expression in catecholamine neurons is reported. However, it still remains unclear which of these changes are beneficial (protective) adaptive responses mediating resilience to stress and which are detrimental leading to vulnerability to the many stress-related disorders. The release of norepinephrine from sympathetic nerve endings is related directly to sympathetic nerve activity; an estimate of norepinephrine in the vascular circulation may be an accurate indicator of that sympathetic nerve activity. Plasma catecholamines can be assayed by radioimmunoassay technique as per the method of Peuler and Johnson [48]. Plasma catecholamines can also be routinely measured using high-performance liquid chromatography (HPLC) with electrochemical detection. Sample volumes required are small and procedure is simple, sensitive and reproducible. It consists of a liquid/liquid plasma catecholamine extraction procedure, HPLC separation and electrochemical detection. This method allows the routine assay of plasma catecholamine concentrations within the ranges in both 500 and 50 ul samples.

Plasma corticosterone assay

Corticosterone is a major indicator of stress in rodents. In rat plasma, the predominant adrenal cortical steroid is corticosterone (in humans the predominant steroid is hydrocortisone) and only small volumes are available for analysis. Both corticosterone and hydrocortisone fluoresce in sulfuric acid and are assayed flourimetrically by the method of Silber et al. [49]. Plasma Corticosterone level can also be assayed by High Performance Liquid Chromatography (HPLC). Rat plasma is extracted with methylene chloride, washed with sodium hydroxide and water. The extract is analyzed by HPLC on a C-18 column with ultraviolet absorbance detection at 254 nm. Another method for corticosterone assay is the ELISA method which is based on the competition between corticosterone and corticosterone tracer. The amount of corticosterone tracer that will be able to bind is inversely proportional to the concentration of corticosterone in the well. After incubation at room temperature, the plate is washed to remove any unbound reagents and then Ellman's reagent (which contained substrate to AchE) is added to the well. The product of this enzymatic reaction has a distinct yellow colour and its absorbance is read at 412 nm. The measured optical density is used to calculate the concentration of corticosterone.

Adrenal ascorbic acid content

Stress results in increased weight of adrenals due to the stress-induced adrenomedullary response which leads to increased production of corticotrophic hormone causing increase in weight of adrenals. The adrenal glands contain relatively large amounts of ascorbic acid, which is markedly decreased by stress or injection of ACTH. For their estimation experimental animals (rats) are divided into groups *viz.* control (no stress); stress; diazepam (positive control) and test compound doses. Treatment is given for 15 days. On 16th day rats are immobilized for 5 h. After 5 h rats are sacrificed, blood collected, adrenals removed and weighed. Ascorbic acid content from blood and adrenal glands are assayed. Adaptogens should reverse the hypertrophy of adrenal caused by stress exposure and normalize the depleted adrenal ascorbic acid content [47].

Tests for anabolic activity

Androgens affect the development of secondary sex organs in the male rat (ventral prostate, seminal vesicles). The growth of the ventral prostate, the seminal vesicles and the levator ani muscle is dependent on the presence of male sexual hormones. Weight development of the levator ani is an index of anabolic activity, and weight development of the ventral prostate and seminal vesicles are markers of androgenic activity. Animals are divided into groups *viz.* controls; test drug groups; and testosterone group. After weighing and castration, 7 day drug treatment is given and on 8th day rats are sacrificed and the wet and dry weights of levator ani muscle, ventral prostate and seminal vesicles of all groups are recorded. Adaptogens should increase the wet and dry weight of the organs as compared to controls.

Tests on gastro-intestinal functions

Stress-induced gastric ulceration is one of the most consistent and robust experimental models for evaluating anti-stress agents. It is different from other models of gastric lesions as a strong central component is involved in such gastric mucosal lesion. Such gastric ulcers are due to both physiological and psychological factors, which are crucial for gastrointestinal defense and increased accumulation of acid and pepsin causing auto-digestion of gastric mucosa. Other factors like platelet activating factor, increased gastric motility, vagal over activity, mast cell degranulation and decreased prostaglandin synthesis, ROS generated by metabolism of arachidonic acid may also result in gastric mucosal damage. The rats swimming stress (water immersion stress model) or restraint/cold restraint stress are commonly used experimental paradigms. After stress procedure rats are sacrificed and

adrenals are weighed. The stomach is removed and cut along the greater curvature, washed and examined with a magnifying lens to calculate the ulcer index (severity and no.). An adaptogen should decrease adrenal weight and both the ulcer nos. and ulcer severity.

Tests for immunological functions

Stress-induced immunomodulation is associated with the various pathophysiological changes [6,7]. Such stress induced alterations in immunological parameters are effectively used to assess anti-stress agents. For example, restraint stress (a well-documented experimental model for emotional stress) induced immunomodulation in rats/mice is a very effective model for testing adaptogenic activity and several conventional and herbal agents have shown consistent effects in this model. Exposure to a variety of environmental stressors like hypoxia, pollutants, xenobiotics like pesticides and drugs have been shown to induce complex immunomodulatory changes in rodents and on the basis of such studies possible strategies to counter such effects have been suggested. From a different perspective, depending upon the nature and intensity of the stressor a wide variety of immune parameters can be evaluated viz. innate and adaptive immune responses, inflammatory markers both in *in vitro* and *in vivo* settings. Humoral and cell mediated immune responses to several antigens like sheep RBC, ovalbumin, tetanus toxoid, keyhole limpet hemocyanin (KLH), etc. as well as cellular markers like cytokines (IFN gamma, IL-4, TNF alfa, IL-5, IL-6, IL-8, IL-13, etc. can be assayed by using appropriate techniques like hemagglutination and ELISA methods. In another protocol, cyclophophamide (CYP) induced immune suppression is used to evaluate effects of adpatogenic agents. Rats/mice are divided into groups: control, CYP, CYP+ test compound, test compound. After 10 days treatment, mice are sacrificed on 11th day and spleen and thymus are isolated and weighed. CYP treated mice show decrease in spleen and thymus weights and adaptogen restore the weights to normalcy. Other xenobiotic eg. pesticides induced immune suppressions have also been reported and the above mentioned immune markers can also be evaluated for testing adaptogenic effects of both synthetic and natural (herbal) compounds. Irrespective of the model/experimental protocol used, adaptogens are expected to attenuate stress/xenobiotic induced immune suppression. Translation of such findings by using adaptogens to clinical settings could lead to innovative therapeutic options in several pathophysiological states involving compromised immunological functions.

Tests for haematological and biochemical parameters

Stress-induced haematological changes are known in both clinical and experimental situations and can be assayed by a simple technique, In a conventional protocol, mice are weighed and divided into groups: control receiving cyclophosphamide (CYP); compound+CYP; compound. Blood is collected from caudal vein on 7th, 14th, 21st and 28th day. The various parameters assessed are total WBC count and RBC count (haemocytometer); hemoglobin content; packed cell volume; lymphocyte count. Adaptogens should reverse the CYP-induced suppression of RBC, WBC; hemoglobin content, packed cell volume and lymphocyte count. Standard biochemical markers, e.g. Glucose, kidney function parameters, liver function parameters, electrolytes, etc. can routinely be assayed by using established biochemical methods or by assay kits. Such reversal of stress induced changes in hematological and biochemical parameters by adaptogens has also been observed in other experimental models.

Tests for oxidative stress markers

Generation of free radicals and imbalance between pro- and anti-oxidant forces result in oxidative stress. Free radicals and reactive oxygen species can target lipids, proteins and DNA and initiate or result in disease states. Several biomarkers for oxidative stress are used to assess the impact of a variety of external and internal aversive stimuli on the organism *viz.* malondialdehyde and isoprostanes (for lipid peroxidation), protein carbonyl and nitrotyrosine (for protein damage), 8-hydroxy deoxy guanosine (8-OH DG, for DNA damage), superoxide dismutase, catalase, reduced glutathione, Nrf-2 factor (for antioxidant status), etc. Oxidative stress markers are also altered in stressful situations and both experimental and clinical studies have indicated this [6]. Several assays for such markers can be performed and the ability of adaptogens to reverse these alter these parameters are assessed. Both *in vivo* and *in vitro* test are commonly performed. Some important assays are briefly discussed below :

Lipid peroxidation: Malondialdehyde (MDA) the organic compound [$CH_2(CHO)_2$] is widely used as oxidative stress biomarker in biomedical research. Lipid peroxidation is measured spectrophotometrically as 2-thiobarbituric acid-reactive substance (TBARS) in blood plasma following the method of Ohkawa et al. [50]. TBARS values are expressed as MDA equivalents. 1,1, 3,3-tetramethoxypropane (TMP) is used as the standard.

Reduced glutathione: This has been suggested as a donor of y-glutamyl groups in general amino acid transport. Reduced glutathione (GSH) is measured according to the method of Nandi and Chatterjee [51] using trichloroacetic acid, phosphate buffer (pH 8.4), 5'5-dithiobis (2-nitrobenzoic acid) and double distilled water. The absorbance read at 412 nm within 15 min. Result expressed as µmol/g tissue.

Superoxide dismutase (SOD): SOD assay in erythrocytes is done in Tsuchihasi extract of erythrocyte haemolysate. The red cells are haemolysed with 3 volumes of cold glass-distilled water. The amount of haemoglobin (Hb) present in the erythrocyte haemolysate is estimated. Ninety-five to 98% of pure SOD added to erythrocyte haemolysate could be recovered in the Tsuchihasi extract described above [52].

Ferric reducing/antioxidant power (FRAP) assay: The chain reaction induced by free radicals can be terminated by electrons donated by an antioxidant. The FRAP assay is a method used to investigate the electron-donating ability of an antioxidant. The principle of the FRAP assay is that electrons offered by an antioxidant will convert the Ferric-TPTZ (Tri PyridylTriazine) complex into a blue colored Ferrous-TPTZ complex, making the antioxidant potential proportional to the FRAP value. Results expressed as FRAP power $mMFe^{2+}$/dry wt. of extract. A lower absorbance value indicates better antioxidant potential of the test sample [53].

DPPH (2,2-diphenyl-1-picryl hydrazyl) scavenging test: The radical scavenging activities of the plant extracts against 2,2-Diphenyl-1-picryl hydrazyl radical is determined by UV spectrophotometry at 517 nm. Vitamin C used as the antioxidant standard at concentrations of 0.02, 0.05, 0.1, 0.2, 0.5 and 0.75 mg/ml. The radical scavenging activity is calculated using the following formula: % inhibition = $\{[A_b - A_a] / Ab\} \times 100$, where A_b is the absorption of the blank and A_a is the absorption of the extract [54].

Iron chelating analysis: Under normal physiological conditions, the ferrous ion is capable of initiating free radical production via the Fenton reaction. Iron chelation prevents free radical production. $FeCl_2$+adaptogen extract+PBS buffer. Reaction is started by adding ferrozine solution and incubated for 10 min at RT. Absorbance (λ=562 nm) measured. Iron chelating potential is expressed as EDTA mg/ml. A lower absorbance value indicates a better ferrous ion chelating ability of the test sample [55].

Hydrogen peroxide scavenging potential: It measures decrease in absorbance due to oxidation of H_2O_2. Absorbance is measured at 230 nm at 0 min and after 60 min. Concentration of H_2O_2 (mM) is determined by using standard curve. Adaptogen should have higher Hydrogen Peroxide scavenging value [55].

Polyphenol analysis: Adaptogen extract is diluted with 80% ethanol. Extract solution with Folin-Ciocalteu reagent and Na_2CO_3 buffer are incubated. After incubation at room temperature, the absorbance (λ=620 nm) in all samples is measured [56].

Nitrates and nitrites (NOx) assay: Nitric oxide (NO) is a highly labile and reactive molecule and gets quickly converted to the more stable nitrates and nitrites (NOx), which are good markers for NO activity/levels. NOx levels determined by using Aspergillus nitrate reductase coupled with NADPH and FAD to convert all nitrates present in sample into nitrites, followed by addition of Griess reagent, for color development and read at 540 nm. Using Standard curves of known concentration of Sodium nitrate and data expressed as NOx/mg protein [57].

Tests for cellular and molecular markers

Heat shock proteins: Recently, several cellular and molecular mechanisms for stress have been defined and their importance as markers of stress susceptibility and adaptation has been highlighted. Maintenance of homeostasis requires tightly regulated and controlled proliferation of cells and their deaths by signal transduction pathways that transmit information to the effectors molecules. Heat shock factors transmit such information for the synthesis heat shock proteins (Hsp). Among various Hsps, Hsp 70 is synthesized at high amounts and is present in the cytosol, nucleus and endoplasmic reticulum playing a protective role against various stress induced insults. It is not usually detectable under normal conditions but is specifically induced in response to a variety of stressors, and is often regarded as a diagnostic cellular marker for stress. The stress proteins of the Hsp70 family function as chaperones, interacting transiently with many proteins and preventing its denaturation in an ATP-dependent manner to protect cells from cell death. Apart from hyperthermia, alterations in the intracellular redox environment, exposure of heavy metals and drugs, various other aversive situations can induce Hsp expression which results in stress tolerance and cytoprotection against stress-induced cellular/molecular damage. Quantitative determination of Hsp 70 (pg/ml of 10% brain homogenate) assays is carried out by ELISA using commercially available kits. The amount of Hsp in each sample was determined by interpolating OD values from Hsp concentration standard curve [58].

Nuclear factor E_2-related factor 2 or Nrf-2: Nrf2 is a novel nuclear transcription factor and is a positive regulator of the human Antioxidant Response Element (ARE) that drives expression of antioxidant enzymes. It is widely distributed in tissue and cell types studies and the role of Nrf2 and Nrf-2 regulated genes be involved in antioxidant-induction and synthesis [59]. The main function of Nrf2 is to activate the cellular antioxidant response by inducing the transcription of a wide array of genes that are able to combat the harmful effects of extrinsic and intrinsic insults, such as xenobiotics and oxidative stress. Nrf2 plays a key role in maintaining redox homeostasis via its interaction with a cysteine-rich protein Kelch-like ECH-associated protein 1 (Keap1). In resting cells, Nrf2 and Keap1 form a tight complex, which is targeted for degradation by proteasomes. Under oxidative stress, Nrf2 is released from the Nrf2/Keap1 complex and translocates to the nucleus where it is able to induce the expression of a battery of genes encoding diverse cytoprotective proteins, including antioxidative enzymes, anti-inflammatory mediators, and proteasomes. As a result, Nrf2 has traditionally been regarded as the cell's main defense mechanism and a major regulator of cell survival. Nrf-2 expression declines with age and leads to dysregulation of anti-oxidant defense systems and Nrf2 activation can modulate the expression levels of hundreds of gene products that can affect oxidative stress and the related pathophysiological states. Activation of the Nrf2 defense response has been shown to protect against neurodegenerative diseases, aging, diabetes, photo-oxidative stress, cardiovascular disease, inflammation, pulmonary fibrosis, acute pulmonary injury and cancer. Thus, Nrf-2 or signaling pathways leading to it may be a potential target for development of anti-oxidants. Nrf2 Transcription Factor Assay is a non-radioactive, colorimetric method for detecting specific transcription factor DNA binding activity in nuclear extracts.Nrf-2 gene expression studies (by RT-PCR method) can also act a good assay techniques for this novel cellular marker for stress.

Conclusion

The increasing incidence of life style disorders highlight the importance of effectively dealing with stress and stressful stimuli. Adaptogens are generally targeted at interactive stress mechanisms and irrespective of their site/mechanism of action, they effectively attenuate stress responses and their impact on health and disease. Several of these agents are present in medicinal plants and their preparations. Exploring the therapeutic value of such adaptogens through validated methods could result in the development of anti-stress agents which could improve the coping capabilities against all kinds of stressors. As the conventional synthetic remedies have disturbing untoward effects, there is a continuous need for search of new herbal cures of various stress related disorders.

Conflicting Interests

The authors do not have any conflicting interests in this study.

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