Anti-stress Effects of Tunisian Cymbopogon schoenanthus L. Ethanol Extract and Some of Its Active Compounds

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Abstract: Many antioxidant rich phytochemical extracts are attracting interest as stress adaptogens. In this regard, several plants are used traditionally and clinically for the management of neurologic disorders. Here, the anti-stress properties of the ethanol extract of medicinal plant Cymbopogon schoenanthus (CSEE), which grows wild in Southern Tunisia, were investigated, as well as the effects of some of the extracted active compounds on H₂O₂-induced cytotoxicity, overproduction of reactive oxygen species (ROS) and adenosine triphosphate (ATP) depletion in human neuroblastoma SH-SY5Y cells. Whereas fluorescence intensity due to DCFH-DA (a marker of ROS production) in H₂O₂-treated SH-SY5Y cells was greater than that in untreated control cells, pretreatment with CSEE and its active compounds ameliorated H₂O₂-induced ROS production. Moreover, H₂O₂ at 150 µM significantly decreased intracellular ATP levels in SH-SY5Y cells, and pretreatment with CSEE and its active compounds buffered this effect. These in vitro results demonstrate the potential of CSEE to protect against stress-associated disorders.

Key words: ATP levels, H₂O₂, medicinal plant, reactive oxygen species, stress.

1. Introduction

Stress is a part of everyday life, and myriad scientific, psychological, medical and even social experts work toward defining and reducing its causes and deleterious effects. Stress alters many physiologic responses, sometimes leading to pathologic outcomes [1]. For example, various stress paradigms significantly affect learning and memory functions and intensify fear memory in mice [2, 3]. Stress-induced effects likely arise through a complex interaction of various factors and mechanisms, including decreases in the levels of central neurotransmitters, neurohormonal factors [4] and neurotrophic factors [5], and increases in free radical generation and oxidative damage in the central nervous system [6]. Furthermore, a variety of disease states, including ischemia-reperfusion injury and hyperoxia, are associated with increased mitochondrial oxidant generation, mitochondrial oxidative injury or ATP depletion [7, 8].

The central nervous system consumes about 20% of energy in the body, using glucose as its main source of fuel [9]. Synapses within the brain are thought to be primary sites of ATP consumption. In addition, ATP is an important extracellular signaling molecule that is released from cells during particular physiologic and pathologic processes, such as neurotransmission, hypotonic stress and cell injury due to inflammation and tumor necrosis [10, 11].

Free radicals are molecules that possess unpaired electrons and arise as necessary intermediates in mitochondrial metabolism. However, some radicals or oxidants, when produced outside these tightly controlled biochemical pathways, can induce specific modifications to biomolecules, thus altering their functions and so contributing to the development of
disease [12]. Whereas a low level of reactive oxygen species (ROS) promotes cell proliferation through the activation of growth-related signaling pathways in normal cells, the biological consequences of excess mitochondrial ROS production are likely substantial. In particular, hydrogen peroxide (H$_2$O$_2$) is one of the main ROS produced during the redox process. H$_2$O$_2$ enhances carcinogenesis and cancer progression [13], and can cause lipid peroxidation and DNA damage, thus inducing apoptosis in many different cell types [14]. In this context, there is growing interest in the search for novel compounds for the treatment of stress, which is considered as a very harmful condition that contributes to numerous pathologies, including cancer, allergy, cardiovascular abnormalities and skin diseases [15-18].

Recent developments in technology and several chemical analyses have revealed that the health-promoting biologic properties attributed to many plant species are in fact due to active compounds that are produced during secondary vegetal metabolism (for example, polyphenols, flavonoids, tannin, essential oils). These secondary metabolites have a wide spectrum of biological activities, including potential anti-inflammatory, anticancer, anti-allergic and anti-stress effects [19-23]. Despite advances in the development of synthetic products, natural medicine is a valuable field of research, because the biological diversity of nature yields a wide range of bioactive molecules [24].

Humankind has long made use of plants to alleviate suffering and disease. For example, herbal medicines are an important part of the culture and traditions of the African continent, where approximately 122 drugs originating from 94 species have been discovered through ethnobotanical leads [25]. Belonging to the family Poaceae (Gramineae), the genus Cymbopogon comprises 56 species [26], which are often aromatic and are distributed widely throughout the Mediterranean area. The focus of the current study, *C. schoenanthus*, is a sub-spontaneous grass and aromatic culinary herb of Southern Tunisia, where it is known locally as “El bekherai.” This plant is common in north and west of tropical Africa, the Arabian Peninsula and Egypt, where it is used in several preparations of meat and salad and is served with tea because of its pleasant aroma [27]. As a medicinal plant, *C. schoenanthus* is used for the treatment of rheumatism and fever [28], as a diuretic and insecticide (it is active against termites and the bruchid beetle *Callosobruchus maculates*) [29] and as a poultice to cure wounds in dromedaries [30]. In various North African countries, *C. schoenanthus* is used as an anti-anorexic and appetite stimulant, an anti-abortive, an anti-convulsive and sedative preparation and a digestive or laxative agent [31].

In this paper by using an *in vitro* bioassay, the anti-stress effects of the ethanol extract of *C. schoenanthus* (CSEE) and some active compounds by focusing on their role in protecting SH-SY5Y cells (a human neuroblastoma line) against H$_2$O$_2$-induced cytotoxicity, ROS production and ATP depletion were investigated.

2. Materials and Methods

2.1 Cell Culture

Human neuroblastoma SH-SY5Y cells were cultured in 1:1 mixture of Dulbecco’s minimum essential medium (Sigma, USA) and Ham’s F-12 nutrient mixture (Sigma, USA). This mixture was supplemented with 15% (v/v) fetal bovine serum (Sigma), 1% (v/v) non-essential MEM amino acids and 1% penicillin (5,000 μg/mL)-streptomycin (5,000 IU/mL) solution (ICN Biomedical). The cells were cultured in 100 mm dishes and passaged at 80% confluence twice weekly by trypsinization using 0.25% trypsin-EDTA (Sigma). In addition, the medium was changed every other day, and the cells were incubated at 37 °C (5% CO$_2$).

2.2 Determination of Cell Viability

Cells were seeded in 96-well plates at a density of 2
× 10^4 cells/well, allowed to attach for 24 h and subsequently treated with various concentrations of H_2O_2. After an additional 24 h of incubation, cell viability was evaluated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL in PBS), as described previously [32]. Briefly, 10 µL of MTT solution was added to each well, and the plate was incubated for 6 h at 37 °C in a 95% humidified air with 5% CO_2 incubator. Then 100 µL 10% (w/v) sodium dodecyl sulfate was added to each well, the plate was incubated overnight and the absorbance at 570 nm was determined. The control wells, which contained cultured cells with medium only, were considered to have 100% cell viability; otherwise, viability was reported as a percentage of the control value. For each experiment, each treatment was performed in triplicate.

2.3 Determination of ROS Production in H_2O_2-Stressed SH-SY5Y Cells

Widely used to evaluate cellular oxidative stress, the lipophilic and non-fluorescent compound dihydrodichlorofluorescein diacetate (H2DCF-DA) passes through the plasma membrane, is de-esterified to a hydrophilic alcohol (H2DCF), and then is oxidized to fluorescent 2',7'-dichlorofluorescein (DCF) through a process generally considered to involve ROS [33]. To assess the effect of CSEE on the production of ROS in H_2O_2-stressed cells, SH-SY5Y cells were seeded at 2 × 10^4 cells/well in 96-well plates and allowed to attach for 24 h. The cells then were treated with CSEE for 24 h. After 24 h, cells were treated with H2DCF-DA for 60 min and then treated with 150 µM H_2O_2 for 60 min or 30 min, after which the fluorescence in each well was measured by using a multi-detection microplate reader (PowerScan HT, Dainippon Pharmaceutical) at 480/530 nm (excitation/emission), and the activity was reported as a percentage of that of the untreated control.

2.4 Measurement of Intracellular ATP Content

Determination of intracellular ATP levels was based on the luciferin-luciferase system. SH-SY5Y cells were seeded at 2 × 10^4 cells/well in 96-well plates and allowed to attach for 24 h at 37 °C. The cells were treated with mixtures of gallic acid (Sigma, USA), ferulic acid (LKT Laboratories) and trans-cinnamic acid (LKT Laboratories), which have been identified as components of CSEE, for 24 h. After this incubation, the cells were exposed to 150 µM H_2O_2 for 3 h. Finally, the luminescence in each well was measured by using a multi-detection microplate reader (PowerScan HT, Dainippon Pharmaceutical) at 480/530 nm (excitation/emission), and the activity was reported as a percentage of that of the untreated control.

2.5 Statistical Analysis

All data are expressed as mean ± standard error of mean (SEM) and the significance of differences was calculated by using Student’s t test. Statistical significance was defined as P < 0.05.

3. Results

3.1 CSEE Protecting SH-SY5Y Cells against H_2O_2-Induced Toxicity

To determine the non-cytotoxic concentrations of CSEE, human neuroblastoma SH-SY5Y cells were treated with CSEE at 1.5, 15 and 150 µg/mL for 48 h. CSEE did not affect SH-SY5Y cells viability at 1.5 µg/mL and 15 µg/mL, and decreased viability only slightly and non-significantly at 150 µg/mL (data not shown). To obtain an appropriate H_2O_2 concentration for use in later assays, SH-SY5Y cells were treated for 24 h with H_2O_2 at concentrations that ranged from 50 µM to 250 µM. Preliminary experiments revealed that 150 µM H_2O_2 reduced the viability of SH-SY5Y cells by 40% after 24 h exposure (Fig. 1), and an H_2O_2 concentration of 150 µM was selected to evaluate the protective effects of CSEE on cell viability.
Anti-stress Effects of Tunisian Cymbopogon schoenanthus L. Ethanol Extract and Some of Its Active Compounds

Fig. 1  SH-SY5Y cells seeded at $2 \times 10^4$ cells/well in 96-well plates for 24 h incubation and then treated with H$_2$O$_2$ (0 (control), 50, 100, 150, 200 and 250 µM) for 24 h.

Cell viability was determined by using MTT assay. Each bar represents the mean ± standard deviation of three independent trials.

*: significant difference at $P < 0.05$; **: significant difference at $P < 0.01$ vs. control group by Student’s $t$ test.

Pre-treatment of SH-SY5Y cells with CSEE (7.5, 15 and 30 µg/mL) for 12 h or 24 h significantly and dose-dependently improved cell viability in H$_2$O$_2$-treated cells compared with that of CSEE-untreated control cells.

3.2 Measurement of Intracellular ROS Production

The effect of CSEE on ROS production in H$_2$O$_2$-treated SH-SY5Y cells was estimated by using the fluorescent dye H2DCFH-DA. The fluorescence intensity of cells treated with 150 µM H$_2$O$_2$ (vehicle-only control) was increased ($P < 0.01$) to 147% and 168% of that of untreated controls after 60 min and 30 min of incubation, respectively (Figs. 2a and 2b). In comparison, pretreatment of the cells with CSEE (7.5, 15 and 30 µg/mL) decreased ($P < 0.01$ for all comparisons) the H$_2$O$_2$-induced accumulation of ROS relative to that in CSEE-untreated cells (Fig. 2).

3.3 Effects of Mixtures Components of CSEE on H$_2$O$_2$-Induced ATP Depletion in SH-SY5Y Cells

The results showed that exposure to 150 µM H$_2$O$_2$ significantly decreased the intracellular ATP level in SH-SY5Y cells to 70% of that in untreated control cells (Fig. 3). To assess the effects of various concentrations of three components of CSEE—gallic acid, ferulic acid and trans-cinnamic acid on intracellular ATP levels in SH-SY5Y cells, four mixtures of these compounds (M1, M2, M3 and M4) were prepared (in each mixture, at least one or two compounds were mixed). Whereas M1 and M2 had no effect on ATP production, pretreatment with M3 maintained the ATP levels of H$_2$O$_2$-stressed SH-SY5Y cells at 90% of those in vehicle-only control ($P < 0.05$). Pretreatment with M4 also yielded a protective effect on intracellular ATP levels, which were 80% of those of the controls.

4. Discussion

Psychiatric conditions, especially stress and depression, are among the most common mood-associated pathologies treated with complementary and alternative therapies [34, 35]. This prevalence is associated with a worldwide increasing trend of coupling primary
healthcare with traditional medicine, which offers a “green image,” cultural significance and accessibility to all societal categories [36]. In this regard, phytochemicals, botanicals and plant extracts are gaining a lot of interest as complementary supplements to mitigate oxidative stress.

Chronic exposure to stress is well known to promote free radical generation and increased ROS levels in the body [6], and oxidative stress is a key factor in neuronal cell death and damage [37]. ROS

![Graph](image1.png)

**Fig. 2**  H$_2$O$_2$-induced ROS production in SH-SY5Y cells incubated with CSEE (0, vehicle only, 7.5, 15 and 30 µg/mL) for 24 h and then exposed to 150 µM of H$_2$O$_2$ for 30 min (a) or 60 min (b).

Results are given as means ± standard deviation of three independent experiments; each experiment contained triplicate samples.

**:** significant difference at $P < 0.01$ vs. untreated control group; **: significant difference at $P < 0.01$ vs. vehicle-only control (cells treated with H$_2$O$_2$ only) by Student’s $t$ test.
Fig. 3  Effects of H$_2$O$_2$-induced ATP depletion in SH-SY5Y cells incubated with mixtures of compounds identified in CSEE (M1-M4) for 24 h and then exposed to 150 µM H$_2$O$_2$ for 3 h. Results are given as means ± standard deviation of three independent experiments; each experiment contained triplicate samples. **: significant difference at $P < 0.05$ vs. untreated control group; *: significant difference at $P < 0.05$ vs. vehicle-only control; **: significant difference at $P < 0.01$ vs. vehicle-only control by Student’s t test.

Fig. 4  Schematic diagram illustrating possible effects of CSEE on ROS and ATP production in H$_2$O$_2$-stressed SH-SY5Y cells.
are generated as a result of normal intracellular metabolism in mitochondria and peroxisomes and from a variety of cytosolic enzyme systems in animal and human cells [38, 39]. In addition, at low concentrations, ROS have several beneficial and necessary roles, including those in detoxification reactions accomplished by cytochrome P-450 complexes, the elimination of cancerous cells through the activation of apoptosis, cellular responses against infectious agents, cellular signaling pathways and the induction of mitogenic responses [40]. However, overproduction of ROS can induce harmful intracellular effects associated with various neurologic diseases and aging [39]. Moreover, the reactions of excess ROS with macromolecules can lead to DNA mutations, changes in the structure and function of proteins and peroxidative damage of cell-membrane lipids [41]. The ROS (H2O2) can diffuse through cell membranes and other organelles to react with various intracellular targets, trigger lipid peroxidation and oxidize proteins and DNA [42]. Furthermore, several studies have shown an increase in ROS production following H2O2 treatment [13, 43].

Given these effects of stress on ROS, antioxidant therapy might be an effective strategy to protect the human body against stress-mediated pathologies.

Most studies dealing with natural antioxidants have focused on the action of flavonoids. In addition to flavonoids, polyphenols, such as caffeic acid, ferulic acid and ellagic acid, occur at high concentrations in several foods and have antioxidant potential [44]. In this study, an increase of ROS and a reduction of ATP level was noticed when cells were exposed to H2O2. As shown in Fig. 4, the treatment of cells with C. schoenanthus prevented these disruptions and reduced the ROS production causing a decrease in HSPs expression genes.

5. Conclusions

It can be confirmed here that the ROS (150 µM H2O2) had deleterious effects on the viability, ROS generation and ATP production of SH-SY5Y cells, a human neuroblastoma line. Through in vitro model to assess the potential neuroprotective effects of CSEE and three of its polyphenol components—gallic acid, ferulic acid and trans-cinnamic acid on H2O2-stressed cells, findings demonstrated that CSEE and some of its components alleviated the toxic effects of H2O2 exposure by reducing ROS production and increasing cell viability and intracellular ATP levels in the model system. The mechanisms underlying the anti-stress effect of CSEE, as well as the component proteins that accomplish this effect, are unknown but may be related to the ability of phenolic acids (e.g., gallic acid, ferulic acid, and trans-cinnamic acid) to prevent a decrease in ATP levels after H2O2 exposure. Future studies will be needed to investigate the specific effects of gallic acid, ferulic acid and trans-cinnamic acid on the activities of antioxidant enzymes in H2O2-stressed cells.

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Anti-stress Effects of Tunisian Cymbopogon schoenanthus L. Ethanol Extract and Some of Its Active Compounds


