IN-VITRO MODELS: RELEVANCE TO NEURODEGENERATION IN DIABETIC NEUROPATHY

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ABSTRACT

Objective: The aim of this study was to analyze antioxidant effect of geraniol (GE) in different in vitro models.

Methods: Initially, the antioxidant activity of GE was assessed by diphenyl picrylhydrazyl radical (DPPH) assay. The modulatory effect of GE against 2,2’-azobis(2-amidinopropane) dihydrochloride induced lipid peroxidation in rat brain regions (cortex and cerebellum) and sciatic nerve (SN) homogenates was determined. Further, the effect of GE was assessed against hyperglycemia-induced oxidative stress (OS) in SHSY5Y, a human neuroblastoma cell line.

Results: GE proved to be a good scavenger of DPPH free radical (inhibitory concentration 50% [IC50] value = 663 nmol) and could lower the lipid peroxidation levels in rat brain tissue and SN homogenates (25-40%). Further, it rescued the SHSY5Y cells from hyperglycemia-induced death. Co-exposure of GE with the IC50 level of glucose (100 mM) lowered the levels of reactive oxygen species, hydrogen peroxides and 3-nitrotyrosine levels with concomitant elevation in the glutathione levels (about two folds).

Conclusion: Collectively from these findings and other studies previously conducted (from our lab and others) emphasize the potential benefit of GE against oxidative stress (OS), a progressive pathological feature of neurodegenerative disorders.

Keywords: Geraniol, Antioxidant, In vitro studies, Diabetic neuropathy.

INTRODUCTION

Oxidative stress (OS) has been understood as one of the prominent features leading to the progression of diabetic neuropathy (DN) and other neurodegenerative disorders (NDD) [1,2]. Development of higher levels of reactive oxygen species (ROS) and reactive nitrogenous species (RNS) is evident in the nervous tissue in various NDD such as Alzheimer’s disease (AD) and Parkinson’s disease (PD). These reactive species include hydroxyl radical, superoxide, nitric oxide, hydrogen peroxide, and peroxynitrites. Overproduction of ROS/RNS, with a concomitant, compromised antioxidant defense system results in significant protein oxidation, lipid peroxidation and nucleic acid oxidation [1]. These oxidized molecules with hampered structure-function lead to various cellular processes including inflammatory response, mitochondrial dysfunction, and possibly apoptosis in NDD [2-4]. DN is a common neuro-degenerative complication with no definite pharmacological solution because of its multifacultural etiology [5].

In this scenario, plant-derived compounds with multiple target might play a role in drug discovery and development. A number of studies have demonstrated potential health-promoting properties of natural products as therapeutics for NDD [6-8]. Several epidemiological reports have documented the influence of dietary habits on lower incidence of NDD. In particular, a significant positive correlation between the consumption of polyphenolic phytochemical-rich foods and the prevention of certain neurological diseases, has been emphasized. Many bioactives such as eugenol, bacopasides, withanolide, and curcumin have been investigated for their neuroprotective function in various NDD models [9-11].

Geraniol (GE) is a bioactive belonging to the class of monoterpenoids [12]. It is found as an important constituent of essential oils of various aromatic herbs. It has a pleasant aroma and is characteristic of rose oil and citrus fruits [12,13]. It is also found in differential amounts in several herbs such as coriander and allspice. It has applications due to its aroma in food and beverage industries as a flavoring agent. It is also found in a wide range of cleansing products and cosmetics for its appealing odor [13,14]. In the recent past, several studies have recognized the pharmacological potential of GE in different models [15-17]. Its pharmacological property is by virtue of its antioxidant and anti-inflammatory properties [18,19].

However, phytochemicals in general and polyphenols, in particular, can also exert pro-oxidant activities under certain experimental conditions [20]. To date, neither pro-oxidant nor antioxidant activities have yet been clearly established to occur in humans. The mechanism through which such dietary supplementation may diminish deleterious effects taking place during degenerative processes of a given disease is not clearly understood. In this study, an attempt has been made to investigate the antioxidant potential of GE in brain and sciatic nerve (SN) homogenates. This was followed by the investigation of the modulatory potency of GE in SHSY5Y, neuroblastoma cell line, under hyperglycemic condition simulating DN. These studies were designed based on the data pertaining to the modulatory effects of GE obtained in in vivo studies from the same lab earlier [16,21].

METHODS

GE, diphenyl picrylhydrazyl radical (DPPH), 2,2’-Azobis(2-amidinopropane) dihydrochloride (AAPH), KRBs reagent, thiobarbituric acid (TBA), trichloroacetic acid, glacial acetic acid, sodium nitroprusside, Griess reagent, hydrogen peroxide, Lowry’s reagent, and FC reagent were purchased from Aldrich-Sigma and SRL. All other chemicals and solvents were of analytical grade and were purchased from local suppliers of manufacturers such as Merck, Hi-media, Sigma and SRL.
Free radical scavenging potency of GE in chemical systems

The antioxidant potential of GE was assessed employing following in vitro methods as described earlier [9].

**DPPH radical scavenging assay**

The DPPH free radical scavenging assay was performed by incubating aliquots of GE (final concentration in the reaction mixture in the range of 0-1600 nmol) with 100 µL DPPH (0.1 mM) for 30 min. The absorbance was measured at 517 nm using an enzyme-linked immunosorbent assay plate reader (molecular devices E750). The assay was performed in three replicates for each concentration. The percent DPPH radical scavenged was calculated and expressed as inhibitory concentration 50% (IC_{50}) value. IC_{50} value was calculated statistically using non-linear regression (dose vs. inhibition).

**Modulatory effect of GE against AAPH-induced lipid peroxidation**

Adult male rats were sacrificed using general anesthesia. Brain and SN were dissected and brain regions, viz., Ctx and SN were separated on ice. Ctx and SN were homogenized (10% w/v) separately in ice-cold phosphate buffered saline (0.1 M, pH 7.4, 0.8% NaCl) centrifuged at 800 × g (10 minutes, 4°C) and the supernatant was collected and freshly used for the in vitro analysis. SN was minced and homogenized under ice in 0.1 M tris-HCl buffer of pH 7.4, and the cytosolic fraction was obtained by centrifuging the samples at 2,000 × g. Initially, tissue homogenates (Ctx, Ctx, and SN) were incubated (1 hr, 37°C) with AAPH (50, 100, and 200 µM) in vitro in a final volume of 500 µL made up using Krebs-Ringer bicarbonate solution as described earlier and the induction of lipid peroxidation was measured. To assess the potency of GE to inhibit AAPH-induced lipid peroxidation, tissue homogenates challenged with AAPH (50 µM) was co-incubated with or without different concentrations (50, 100, and 200 nmol) of GE for 1 hr. Separate GE aliquots were maintained without AAPP to monitor any per se effect. After the incubation period, the extent of formation of lipid peroxides was quantified as levels of malondialdehyde (MDA) by measuring TBA reactive substances.

**Cell culture experiments: In vitro model of hyperglycemia in SHSY5Y cells**

**Induction of hyperglycemia**

SHSY5Y, a human neuroblastoma cell line, was maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum and pen-tre. The growth conditions include a humid atmosphere of 5% CO₂ and 95% O₂, at 37°C. Cells were plated in 96-well plates at a density of 5×10⁴ cells/well. Cells were exposed to different concentrations of glucose (Glc; 25-300 mM) for 24 hrs. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyloxazoliumbromide] assay was carried out to determine cell survivability [22]. The yellow tetrazolium MTT gets reduced by metabolically active cells, in part by the action of dehydrogenase enzymes resulting in the formation of purple formazan crystals. These intracellular formazan crystals were then solubilized in SDS-DMF buffer (45% DMP in distilled water and 10% SDS, pH 4.7). It was quantified spectrophotometrically at 570 nm, and percent cell survivability was calculated against control (using OD as a measure).

**Effect of GE on cell survivability**

A 10 mM stock solution of GE was prepared in 95% ethanol, while all the working standards were prepared in the culture media. Initially, cells were plated in 96 well plates at a density of 5×10⁴ cells/well containing different concentrations (1-100 µM) of GE for 24 hrs. Cell survival was determined by MTT assay to ascertain the non-toxic concentrations. For further experiments, only selected concentrations were employed, to assess the potential to modulate glucose-induced cellular aberrations and cell death.

**Modulatory effects of GE on glucose-induced OS**

SHSY5Y cells were co-exposed to sub-lethal concentrations of GE and 100 mM of glucose (IC_{50}, concentration) for 24 hrs. Cell survival and protection rendered by the active against glucose-induced cell death was determined by MTT assay. For biochemical estimations, experiments were carried out only with a functional concentration of 10 µM of GE (i.e., the concentration at which it rendered protection against glucose-induced cell death). Selected markers for OS (generation of ROS, levels of hydroperoxides (HP) and glutathione [GSH]) were determined post 24 hrs co-exposure.

**Modulatory effect of GE on ROS levels**

ROS generation was assessed using 2,7-dichloro-fluorescein diacetate (H₂DCFH-DA), a nonpolar compound, which after conversion to a polar derivative by intracellular esterases, rapidly reacts with ROS to form the highly fluorescent compound DCF [23]. Briefly, an aliquot (100 µg protein equivalent) was incubated in Locke’s buffer (pH 7.4; NaCl: 154 mM, KCl: 5.6 mM, NaHCO₃: 3.6 mM, HEPES: 5 mM, CaCl₂: 2 mM, and glucose: 10 mM) containing H₂DCFH-DA (5 mM) for 30 minutes at room temperature. The fluorescent product DCF was measured using a spectrophotometer with an excitation and emission wavelengths of 480 nm and 530 nm, respectively. The ROS generation was calculated from a DCF standard curve and expressed as nmol DCF/minute/mg protein.

**Modulatory effect of GE on HP levels**

HP levels were measured according to a previously described method using FOX 1 reagent with minor modifications [24]. An aliquot of cytosolic (or mitochondrial) fraction (100 mg protein) was added to 1 mL FOX reagent (100 µL xylanol orange: 250 µM ammonium ferrous sulfate; 100 µM sorbitol; 25 mM H₂SO₄) and incubated for 30 minutes at room temperature. The color developed was read at 560 nm in a spectrophotometer. The concentration of HP was calculated using the molar extinction coefficient (ε = 2.2×10⁴ M/cm) and expressed as nmol/mg protein.

**Modulatory effect of GE on GSH levels**

GSH content was quantified based on a fluorimetric method as described previously using o-phthalaldehyde (OPT) [25]. Briefly, an aliquot of the cytosolic fraction was added to formic acid (0.1 M) and centrifuged at 10,000 g for 10 minutes. An aliquot of supernatant (deproteinized) was added to tubes containing buffered formaldehyde (1: 4 (v/v) 37% formalin: 0.1 M Na₂HPO₄). Sodium phosphate buffer (0.1 M; pH 8.0; containing 5 mM ethylenediaminetetraacetic acid) was added to each tube followed by OPT (100 µg/mL). Following incubation for 45 minutes at room temperature, the fluorescence was measured (excitation: 345 nm and emission: 425 nm). Concentration of GSH was calculated from a standard curve and values were expressed as nmol/mg protein.

**Modulatory effect of GE on nitrosated proteins 3-nitrotyrosine (3-NT)**

The extent of protein nitration was estimated in terms of 3NT employing a slot-blot method. In brief, aliquots (15 µg protein) were spotted in triplicates onto a nitrocellulose membrane. The membranes were treated with a blocking buffer containing 2% bovine serum albumin in PBS-Tween 20 (PBST) (PBS, 0.1 M, pH 7.4, containing 0.14 M NaCl and 0.01% Tween-20) for 60 minutes at room temperature and probed with polyclonal anti-3-NT antibody (1:500; origin- Rabbit; Sigma Chemical Co. USA) over the next 60 minutes. The membranes were washed repeatedly in PBST and incubated with ALP-conjugated secondary antibody (1:2000; origin-goat; Merck- Bangalore Genei, India) for 60 minutes. The membranes were developed employing BCIP/NBT reaction. To adjust for protein loading, duplicate membranes were also immune-stained with Anti-β-actin monoclonal antibody (1:2000; Sigma, USA). Optical density on the blots was measured with ImageJ software (NIH, USA). The extent of protein nitration was expressed as arbitrary units [26].

**RESULTS**

Free radical scavenging effect of GE in chemical systems

**DPPH radical scavenging assay**

GE scavenged the free radical DPPH to a yellow colored 1,1-diphenyl-2-picrylhydrazyl in a concentration-dependent manner (Fig. 1). The IC_{50} value was found to be 66.3 nmol by statistical analysis (R²=0.9513).
Modulatory effect of GE against AAPH-induced lipid peroxidation

AAPH exposure resulted in a concentration-dependent increase in lipid peroxidation in different tissues of rat (Data not shown). The elevated levels of MDA (indicative of lipid peroxidation) induced by 50 µM AAPH in different tissues were: Ct: 67.7±6%; Cb: 90.8±3%; SN: 69.4±9%. While GE reduced the extent of lipid peroxidation in Ct at all concentrations tested, in Cb and SN reduction was evident only at 100 nmol concentrations (Fig. 2).

Cell culture experiments: In vitro model of hyperglycemia in SH-SY5Y cells

Effect of glucose and GE on cell survivability

Exposure of SH-SY5Y cells to different concentrations of glucose resulted in a concentration-dependent cell death as evidenced by MTT reduction assay. Based on these previous preliminary studies, for studying the modulatory potency of GE, glucose concentration of 100 mM (IC$_{50}$) was used [27]. Further, exposure of cells to GE per se did not cause any cell death in the concentration range of 5-50 mM. Hence, the concentrations used for studying the modulatory potency of GE against hyperglycemia were in the range of 5-50 µM. GE offered protection (p≤0.05) only at the concentration of 10 µM (Fig. 3).

Modulatory effects of GE on glucose-induced OS

Glucose exposure caused significant induction of OS as evidenced by elevation in ROS (20%) and HP (50%) levels as well as depletion in GSH levels (35%) (Table 1). GE exposed cells exhibited a significant reduction in the endogenous levels of ROS (37%) and HP (27%). While ROS levels were reduced marginally by GE when co-exposed with glucose, the HP levels were normalized (Fig. 4a and b). While no alteration in the endogenous levels of GSH/GSSG with GE exposure was evident, on co-exposure with glucose, the GSH levels were increased two folds (Table 1).

Modulatory effects of GE on 3-NT levels

Glucose exposure to SH-SY5Y cells resulted in a marked increase (46%) in 3-NT levels. While GE reduced the endogenous levels moderately, a marked diminution (about three-fold against glucose per se) occurred when co-exposed with glucose (Fig. 5).

DISCUSSION

The complexity of biochemical pathways in central nervous system and peripheral nervous system in NDD is not fully understood. Nevertheless, OS and inflammatory reactions play a central role in degenerative processes. This necessitates to target the increase in OS by supplementing with antioxidants and/or to fortify antioxidant defense system to keep a check on the progression of different degenerative pathways. This is true of neurodegeneration seen in the case of AD, PD or DN. Several labs including ours have been investigating the possibilities of employing plant based actives against NDD [9-11,28]. The mechanisms through which supplementation of such bioactives may diminish free radical-related diseases like NDD is associated to their multiple biological abilities. This includes their ability to reduce the levels of or the formation of ROS and RNS, concomitantly up-regulating vitagenes, such as members of the heat shock protein (Hsp) family, heme oxygenase-1, and Hsp70 [8,26].

GE has been reported as an effective antioxidant and anti-inflammatory agent in different experimental models from our lab and others [16-19,21,29]. Recently, the antioxidant activity of GE along with other actives has been published with respect to DPPH free radical scavenging activity and superoxide dismutase activity [30]. However,
this study was planned and executed bearing in mind our results in different experimental models of neurodegeneration [16,21]. These studies emphasized the modulatory potency of oral supplementation of GE against acrylamide induced neurotoxicity and DN. In both the in vivo studies, it was evident that GE supplementation could interfere with OS by altering the levels of oxidative markers such as ROS, hydrogen peroxides, nitrates and protein carbonyls in brain regions, and SN. Hence in this study, first the DPPH free radical scavenging capacity was determined and was found to be concentration dependent (correlation: $R^2 = 0.9513$). Further, the homogenates of Ct, Cb, and SN were challenged with AAPH leading to the induction of lipid peroxidation in these high lipid containing nervous tissues. This mimics the condition of high lipid peroxidation levels noted in NDD. The modulatory potential of GE against AAPH induced lipid peroxidation was clearly evident by the reduced levels of MDA in all tissue homogenates tested. This emphasizes the role of GE to reduce lipid peroxidation in the in vivo models studied earlier.

Further, experiments were carried out in media containing high levels of glucose in the SHSY5Y undifferentiated cells simulating diabetic condition. In the case of neuronal cells the glucose uptake is facilitated diffusion, and hence whenever the blood glucose levels are high as is the case of diabetes, the neuronal glucose level is elevated [31,32]. Hyperglycemia leads to the activation of various glucose-mediated pathways such as polyol pathway, AGE formation pathway, activation of protein kinase A and hexosamine monophosphate pathway ultimately culminating to the development of OS, inflammatory reactions and apoptotic process [2,33]. In SHSY5Y cells, hyperglycemic condition induced cell death and elevation in OS markers such as ROS, hydrogen peroxides, and 3-nitrotyrosine [27,34]. GE co-exposure with the IC$_{50}$ level of glucose rescued the cells from death. Further, enhanced levels of ROS and hydrogen peroxide were significantly reduced with GE clearly suggesting its ability to interfere OS related mechanism under high glucose levels. Interestingly, GE also enhanced the depleted levels of GSH with concomitant change (about 20%) in the levels of GSSG (Data not shown). This is in line with our previous findings in streptozotocin model of diabetes in rat where GE significantly altered the oxidative markers in different regions of the brain and SN [16]. Further, antioxidant effect is a key feature which is being investigated by several diabetes researchers and hunt for novel phytochemicals alone or in the form of extracts is going on worldwide [32,35].

CONCLUSION

In this study, the in vitro analysis employing various models exhibited the antioxidant activity of GE. These findings and studies from other labs further emphasizes the potential of GE against free radical mediated deleterious effects under various disease conditions like NDD [18,31,36]. GE is hence proposed to be employed as an adjuvant in the management or prevention of neurodegenerative processes involved in NDD like DN.

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**Table 1: Modulatory effect of geraniol (GE 10 µM) on reduced GSH in SHSY5Y cells co-exposed with glucose (Glc, 100 mM) for 24 hrs**

<table>
<thead>
<tr>
<th>Group</th>
<th>GSH (nmol/ mg protein)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>22.2±0.7</td>
</tr>
<tr>
<td>GE 10 µM</td>
<td>22.5±1.2</td>
</tr>
<tr>
<td>Glc 100 mM</td>
<td>14.9±0.8*</td>
</tr>
<tr>
<td>Glc + GE 10 µM</td>
<td>29.1±0.3*</td>
</tr>
</tbody>
</table>

GSH: Glutathione. Data analyzed by one-way analysis of variance followed by Tukey's test for comparison of means. *Significant against control, #against glucose (Glc, 100mM), p≤0.001.
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REFERENCES


