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Protective effect of *Satureja montana* extract on cyclophosphamide-induced testicular injury in rats

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ABSTRACT

The present study investigated the protective effect of *Satureja montana* extract against cyclophosphamide-induced testicular injury in rats. Total phenolic and flavonoid contents of the extract were 1.03% and 0.34% w/w of dry herb expressed as chlorogenic acid and quercetin, respectively. HPLC analysis identified caffeic, syringic and rosmarinic acids as the chief phenolic acids, and rutin as the major flavonoid in the extract. Oral daily administration of *S. montana* extract (50 mg/kg/day) for 7 days before and 7 days after an intraperitoneal injection of cyclophosphamide (200 mg/kg) restored the reduced relative testicular weight, serum testosterone level and testicular alkaline phosphatase activity, raised the lowered testicular sorbitol dehydrogenase and acid phosphatase activities, and decreased the elevated testicular hemoglobin absorbance. It also attenuated lipid peroxidation, restored the lowered glutathione content, glucose-6-phosphate dehydrogenase, glutathione peroxidase and glutathione reductase activities, and improved total antioxidant capacity. Moreover, *S. montana* extract mitigated testicular DNA fragmentation, decreased the elevated Fas and Bax gene expression, up-regulated the decreased Bcl-2 and peroxisome proliferator-activated receptor-gamma (PPAR-γ) gene expression and normalized Akt1 protein level. Histopathological investigation confirmed the protective effects of the extract. Conclusively, *S. montana* extract protects the rat testis against cyclophosphamide-induced damage via anti-oxidative and anti-apoptotic mechanisms that seem to be mediated, at least in part, by PPAR-γ and Akt1 up-regulation.

1. Introduction

Satureja montana L., commonly known as winter savory or mountain savory, is a perennial shrub (20–30 cm high) with white flowers and small rough leaves belonging to the Lamiaceae family and is native to the Mediterranean region. *S. montana* is an aromatic culinary and medicinal plant [1]. Phytochemical investigations demonstrated the presence of many biologically active constituents in *S. montana* accounting for its medicinal values such as the monoterpenes carvacrol, p-cymene, γ-terpinene and thymol in the essential oil, rosmarinic acid, triterpenes, flavonoids and caffeic acid [2,3]. The whole plant, its essential oil and extracts are used in traditional medicine for their stomachic, digestive, carminative, expectorant, aphrodisiac, bactericidal and fungicidal activities [1]. In addition to being traditionally used as an aphrodisiac remedy, recently, *S. montana* could be considered as a natural remedy for the treatment of premature ejaculation. Furthermore, its reported ability to raise the serum level of testosterone confirms its positive influence on male sexual function [3]. However, to our knowledge, there are no reports regarding a potential protective effect of *S. montana* against testicular injury.

Oxidative stress is a key factor in the etiology of male infertility, and peroxidative damage is currently regarded as the single most important cause of impaired testicular function in a wide range of testicular stresses. Increased testicular oxidative stress can cause

Abbreviations: ACP, acid phosphatase; ALP, alkaline phosphatase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; CP, cyclophosphamide; DAD, diode array detector; FasL, Fas ligand; Fe^{III}-TPTZ, ferric-tripyridyltriazine complex; FRAP, ferric reducing antioxidant power; FSH, follicle stimulating hormone; GC/MS, gas chromatography–mass spectrometry; γ-GT, gamma-glutamyl transferase; G6PD, glucose-6-phosphate dehydrogenase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; H&E, hematoxylin and eosin; Hb, hemoglobin; LDH, lactate dehydrogenase; LH, luteinizing hormone; MDA, malondialdehyde; PI3K/Akt, phosphatidyl inositol-3-kinase/serine threonine kinase; PPAR-γ, peroxisome proliferator-activated receptor gamma; ROS, reactive oxygen species; RT-PCR, real time polymerase chain reaction; SDH, sorbitol dehydrogenase; TAC, total antioxidant capacity; TFC, total flavonoid content; TPC, total phenolic content.

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changes in the dynamics of testicular microvascular blood flow and endocrine signaling leading to an increase in germ cell apoptosis and subsequent hypospermatogenesis [4].

Germ cell apoptosis is a significant process even in conventional spermatogenesis, but the process is up-regulated in testicular stress conditions [5]. There is a prominent role for the mitochondrial pathway in germ cell apoptosis, where associations between pro-apoptotic and anti-apoptotic members of the Bcl-2 family (e.g. Bax and Bcl-2, respectively) are altered, allowing the release of cytochrome c and the eventual activation of a caspase cascade, which ultimately results in DNA fragmentation [6]. The Fas system is also a key regulator of germ cell apoptosis in the testis. This is a pathway involving Fas ligand (FasL) and Fas, members of the TNF superfamily of ligands and receptors [7]. FasL is secreted by Sertoli cells, and its receptor, Fas, is on the germ cell membrane. Fas–FasL binding initiates the intracellular "death domain" pathway, which, like the mitochondrial pathway, eventually leads to DNA degradation via the caspase cascade [4]. Important regulatory pathways of testicular homeostasis also include the peroxisome proliferator-activated receptor-gamma (PPAR-γ) [8] and phosphatidyl inositol-3-kinase/serine threonine kinase (PI3K/Akt) [9] signaling systems.

Cyclophosphamide (CP) is an alkylating agent that has been widely used in the acute treatment of various neoplastic diseases and in the chronic treatment of autoimmune disorders. The major limitation of CP chemotherapy is the injury of normal tissue, leading to multiple organ toxicity mainly in the testes, heart and urinary bladder [10]. The therapeutic and toxic effects of CP are mediated by its active metabolites phosphoramide mustard and acrolein [11]. Phosphoramide mustard acts by the formation of a positively charged reactive intermediate that irreversibly binds to DNA causing intra- and interstrand cross-linkages, leading to DNA strand breaks, the inability to synthesize DNA, and ultimately cell death, which is a major anticancer mechanism of CP. Acrolein, which inactivates the DNA repair protein O6-methylguanine-DNA methyltransferase, also partially contributes to the cellular toxicity of CP [11].

The cytotoxicity of CP to rapidly dividing cells makes the highly proliferative testes a target for the damaging effects of this drug. A number of reports described the adverse effects of CP on fertility in humans and animals. Male patients treated with CP develop oligospermia and azospermia [12]. In rodents, administration of CP leads to decreased testicular weight, transitory oligospermia, decreased DNA synthesis in spermatogonia and protein synthesis in spermatids [13]. Cyclophosphamide-induced testicular toxicity is considered an important model combining major oxidative and apoptotic alterations [14]. Therefore, this model was adopted in the present study as a representative of the deregulated redox homeostasis and germ cell survival/apoptosis occurring in a wide range of testicular stresses.

There are several reports on the benefit of antioxidants in protecting male reproductive system from deleterious effects of reactive oxygen species (ROS) generated during CP exposure [10,15]. Among natural antioxidants, phenolic compounds, such as caffeic acid, rosmarinic acid and the monoterpenes abundant in *S. montana*, are well known for their protective potential against ROS overproduction though direct and indirect antioxidant mechanisms [16,17].

Therefore, the present study was designed to investigate, for the first time, the protective effect of *S. montana* extract against CP-induced testicular injury in rats, following the quantification of total phenolics, total flavonoids and individual phenolic compounds in the extract. This study is an attempt to introduce a relatively safe approach to alleviate the almost inevitable consequence of CP therapy.

2. Materials and methods

2.1. Plant material

Seeds of *S. montana* were obtained from the HEM ZADEN B.V., P.O. Box 4, 1606 ZG Venhuizen, The Netherlands. Seeds were sown in the nursery under the natural conditions of the greenhouse of the National Research Center, Giza, Egypt, on November 25th, 2011. On February 10th, 2012, uniform seedlings were transplanted into the experimental farm of the Faculty of Pharmacy, Cairo University, Giza, Egypt, which represents clay-loamy soil. The fresh herb was collected at the end of July, 2012. The herb was air-dried in a room (under shade) for 3 weeks.

2.2. Preparation of plant extract

A hydroalcoholic extract of *S. montana* was prepared by placing the dried aerial parts of the plant (250 g) in 80% ethanol (Sigma–Aldrich Chemicals Co., St. Louis, MO, USA) containing 1% hydrochloric acid (1 L × 3 times) at room temperature with the aid of sonication. The combined alcoholic extracts were filtered and evaporated to dryness under vacuum at 45 °C. The crude alcoholic extract was subjected to quantitative estimation of the total phenolic and flavonoid contents as well as HPLC analysis.

2.2.1. Determination of total phenolic content (TPC)

The Folin–Ciocalteu method was used to determine total phenolic content (TPC) [18]. The absorbance was measured at 725 nm, and chlorogenic acid (Sigma–Aldrich Chemicals Co., St. Louis, MO, USA) was used as a standard to produce the calibration curve. The results were expressed as chlorogenic acid equivalents mg/100 g of dry herb weight.

2.2.2. Determination of total flavonoid content (TFC)

The total flavonoid content (TFC) was determined according to the colorimetric assay of Chang et al. [19] using a calibration curve with quercetin (Sigma–Aldrich Chemicals Co., St. Louis, MO, USA) as standard. Absorbance was measured at 510 nm. Results were expressed as quercetin equivalents mg/100 g of dry herb weight.

2.2.3. Quantification of phenolic compounds by HPLC analysis

Phenolic acid and flavonoid standards were quantified by preparing in methanol solution (HPLC grade, Merck, Germany), and serial dilutions were carried out by double-distilled water. Various standard concentrations were injected into HPLC system to establish standard calibration curves. Analyses were developed by HPLC system (Agilent Technologies, Waldbronn, Germany, modular model 1200 series instrument), equipped with Eclipse DB-C18 column (5 μm, 4.6 × 250 mm *i.d.*) according to Neo et al. [20]. A mobile phase was prepared consisting of acidified water and acetonitrile in the ratio of 90:10 (v/v), at a flow rate of 1 ml/min. Detection was done using a diode array detector (DAD) at 280 and 320 nm and the chromatographic data analyses were done using Chemstation soft-ware. Quantitation in each gram of sample was carried out using external standard method. The amount of each phenolic compound was expressed as micrograms per gram of dry plant weight (μg/g).

2.3. Animals

Male Wistar albino rats weighing 170–200 g were used for the study. Rats were allowed free access to commercial pellet diet and water throughout the experimental period. All animal experiments were conducted in accordance with the approval of the Ethical Committee for Animal Experimentation at Faculty of Pharmacy,