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Research article

Nephroprotective potential of whey protein concentrate and fennel seed extract against tienilic acid -induced renal dysfunctions in albino rats

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Abstract

The objective of this study was to evaluate the nephroprotective potential of whey protein concentrate (WPC) and methanolic fennel seed extract (FSE) against tienilic acid (a hypotensive drug) -induced renal dysfunctions in albino rats. Sixty rats were divided into six equal groups and treated orally for six weeks as follows: control group; FSE alone (200 mg/ kg/day); WPC alone (0.5 g/kg/day); TA alone (1g/kg/twice a week); TA (1g/kg/twice a week) plus FSE (200 mg/kg/day). TA (1g/kg/twice a week) plus WPC (0.5g/kg/day). TA administration significantly increased serum levels of creatinine, urea and potassium while decreased significantly sodium and corticosterone levels. On the other hand, Na+/ K+-ATPase activity and total antioxidant capacity content in kidney tissue were diminished concomitant with a significant rise in the levels of kidney lipid peroxidation and nitric oxide. Furthermore, serum tumor necrosis factor-α and interleukin-6 levels were significantly elevated while serum calcium was unchanged. The treatment with either WPC or FSE to TA–treated animals significantly protected the kidney tissue against the injurious effects of tienilic acid. They effectively ameliorated most of the changes induced by TA towards the normal values of the controls. It could be concluded that the renoprotective effect of these agents might be through the prevention or scavenging of free radicals. This may be attributed to the high content of antioxidant compounds in WPC and fennel extract.

Introduction

The kidney represents the major control system maintaining the body homeostasis [1]; it is considered as the major organ for filtration, re-absorption and also excretion of drugs or drug metabolites [2]. As a consequence, the kidney is vulnerable to toxic revilement, by various drugs or xenobiotics, and thus nephrotoxicity is one of the major concerns in preclinical safety evaluation. Despite the morphological complexity of the kidney, the renal tubular epithelial cells emerge as one of the most sensitive components in the kidney and are thus highly susceptible to damage [2].

Tienilic acid is a diuretic with hypotensive and marked hypouricaemic properties [3]. Therefore, it has been proposed as the drug of first choice in patients with hypertension who already have gout [4]. The clinical use of tienilic acid is, however, associated with particular side effects such as hepatotoxicity [5-7], and autoimmune diseases [8,9].

Fennel (*Foeniculum vulgare* M, *Apiaceae*) is a well-known mediterranean aromatic small, hardy perennial herb widely used as food and with an established role as herbal remedy [10]. Both infusions and essential oils obtained from the fruits and the aerial parts of the plant are included in the herbalist armamentarium for their dysmenorrhea [11], relaxant [12], analgesic and anti-inflammatory properties [13]. The essential oil showed also antioxidant [13], antimicrobial [14] and immuno-protective activity [15]. The dried, aromatic fruits are widely employed in culinary preparations for flavouring bread and pastry, in candies and in alcoholic liqueurs of French type, as well as in cosmetic and medicinal preparations [16].

Whey proteins, a byproduct of cheese manufacture [17]. It recognized as a valuable food ingredient with important nutritional and functional properties is gaining acceptance

as functional food ingredient. Whey proteins have a high content of sulfur-containing amino acids, which support antioxidant functions [18] owing to the abundance of cysteine or the presence of glutamyl cysteine which facilitate glutathione (GSH) synthesis [19-21]. Feeding whey proteins would prevent aflatoxins-induced liver damage [22]. It would protect also against CCl_4-induced erythrocyte damage [23] and against tienilic acid -induced liver toxicity in rats [24].

Therefore, the objective of this study was to evaluate the renoprotective batteries of two antioxidant and safe agents derived from different natural sources, WPC as animal product and FSE as plant product, against kidney damage induced by tienilic acid in rats as an experimental model for nephrotoxicity.

Experimental

Materials

Fennel (*Foeniculum vulgare*) seeds were obtained from Abd El-Rahman Harraz (Bab El-Khalk zone, Cairo, Egypt). The herb was identified in the Botany Department, National Research Centre. Tienilic acid: (purity 99.7%) was purchased from DAIICHI SANKYO Co., Ltd., Tokyo, Japan. It was suspended in 1% methylcellulose (from Wako Pure Chemical Industries, Osaka, Japan). Whey proteins concentrate (WPC) powder contains 80% proteins was obtained from Davisco Foods International, Inc., Eden Prairie, Minnesota, USA. The WPC was prepared according the method described by Kennedy *et al.,* [25] by mixing with water and stirring with a magnetic stirrer and left in refrigerator overnight to fully hydrate. All other chemicals were of the highest analytical grade available.

Herb extraction

Alcoholic extract was prepared from fennel seeds according to the procedure described by Anwar *et al.*, [26]. Ground dry sample (100 g) was extracted with 1000 ml 80% methanol (80:20, methanol: water, v/v) using an orbital shaker for 8 hours at room temperature. The extract was separated from solids by filtering through Whatman No. 1 filter paper. The remaining residue was re-extracted twice and the extracts were pooled. The solvent was removed under vacuum at 45°C, using a rotary vacuum evaporator and stored at –4°C until used for further analyses. This procedure resulted in an average yield of 11.8 % (w/w) of methanolic extract based on the dry weight.

Experimental design

Adult male Sprague–Dawley (SD) rats weighting 130-150g were obtained from animal house colony, Giza, Egypt. One week of acclamation before the administration, the animals were housed in wire-mesh cages in an air-conditioned room and allowed free access to tap water and commercial rodent chow purchased from Meladco Feed Co (Aubor City, Cairo, Egypt) at the Animal House Lab, National Research Centre, Dokki, Cairo, Egypt.

After one week of acclimation, animals were randomly arranged in six groups (10 rats each) and treated for six weeks as follows: group (1) animals given physiological saline and served as control group, group (2) animals treated orally with WPC (0.5 g/kg/day), group (3) animals treated orally with fennel seed extract (FSE) (0.2g/kg/day) [13], group (4) animals treated orally with tienilic acid (1g/kg twice a week) [6] dissolved in 1% methylcellulose solution, group (5) animals treated orally with tienilic acid (1g/kg twice a week) combined with WPC (0.5 g/kg/day) and group (6) animals treated orally with tienilic acid (1g/kg twice a week) combined with FSE (0.2 g/kg/day).

Blood and tissue sampling

At the end of the treatment period, blood samples were collected from the retro-orbital venous plexus from each animal under diethyl ether anesthesia. Blood samples were left 20 minutes to clot, then centrifuged at 3000 rpm for 10 minutes using cooling centrifuge (IEC centra-4R, International Equipment Co., USA). The sera were separated at once by micro pipette, divided into aliquots and stored at -70°C, until analysis.

After blood collection, all animals were rapidly sacrificed and the left kidney of each animal was dissected out, washed with saline, dried, rolled in a piece of aluminum foil and stored at -70°C until homogenization for tissue biochemical determinations. Samples of the kidneys were weighed (approximately 0.05–0.1 g) and homogenized in phosphate buffer (pH 7.4) to give 20 % (w/v) homogenate and centrifuged at 5000 rpm for 20 minutes. The clear supernatants were drown out, divided into aliquots and stored at -70°C till the determination of the requested biochemical parameter.

Biochemical determinations

An enzymatic procedure was used to determine serum urea level using a kit obtained from Biodiagnostic Co. (Egypt). Serum creatinine concentration was kinetically evaluated using a kit obtained from Biodiagnostic Co. (Egypt). Serum sodium and potassium levels were evaluated using kits purchased from DiaSys GmbH, Germany using MEDICA Easylyte Na/K ANALYZER (USA). Total calcium in serum was estimated by colorimetric method using a kit purchased from Randox Laboratories LTD Co., UK. Serum corticosterone level was determined using ELISA reagent kit purchased from Immunospect, Canoga Park, USA. Renal total antioxidant capacity (TAC) and nitric oxide (NO) levels of the homogenates were determined using the reagent kits produced by Biodiagnostic, Dokki, Giza, Egypt. While kidney level of lipid peroxidation end product,

malondialdehyde (MDA) and Na^+/K^+-ATPase activity were determined according to the modified chemical methods of Ruiz-Larrea [27] and Tsakiris [28] respectively. Serum tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) levels were determined using enzyme-linked immuno-sorbent assay (ELISA) using enzyme immunoassay kits for the quantitative determination of TNF-α and IL6 purchased from Ray Biotech Co., USA.

Statistical Analysis

The obtained data were subjected to one way analysis of variance (ANOVA). The analysis was performed using statistical analysis system (SAS) program software; copyright (c) 1998 by SAS Institute Inc., Cary, NC, USA. Tukey test was used to evaluate the significance between the individual groups at $p \leq 0.05$ [29].

Results and Discussion

Results

Figure (1) shows the effect of tienilic acid administration individually or in combination with fennel seed methanolic extract (FSE) or whey protein concentrate (WPC) on the specific biomarkers of kidney function. Administration of tienilic acid significantly increased serum urea and creatinine concentrations when compared with those of control. The treatment with WPC or FSE alone resulted in no significant effects on these parameters as compared with the normal rats indicating safety of both agents. Administration of WPC or FSE in combination with tienilic acid showed significant reduction in serum urea and creatinine levels compared with tienilic acid-treated group, although their levels still higher than the control values.

Serum potassium, sodium, calcium and corticosterone levels in the different studied groups are depicted in figure (2). Tienilic acid administration significantly decreased sodium and corticosterone concentrations while potassium concentration was significantly increased as compared to those of the control group. Oral administration of WPC in combination with tienilic acid produced significant increase in corticosterone level while it caused insignificant changes in sodium and potassium concentrations as compared to those in the tienilic acid -treated group. On the other hand, administration of FSE in combination with tienilic acid resulted in significant increase in sodium and corticosterone levels but not affect potassium level when compared to those in the tienilic acid -treated group. Figure (2) shows also non significant differences in serum calcium among the different studied groups.

Data in figure (3) show the effects of different treatments on oxidative stress markers and Na^+/K^+-ATPase activity of the animals. It is clearly indicated that tienilic acid increased significantly the levels of renal MDA and NO while it decreased significantly the values of renal TAC level and Na^+/K^+-ATPase activity compared to control group. Treatment with WPC or FSE alone had no significant effect on renal MDA, NO, TAC or Na^+/K^+-ATPase as compared to those in the normal rats. The administration of WPC or FSE succeeded in ameliorating the tienilic acid-induced significant changes in the mentioned parameters and reversed their values toward the values of the controls.

Figure (4) illustrates the effect of different treatments on serum inflammatory markers. Serum TNF- α and IL-6 were significantly increased in the group received tienilic acid alone compared to the controls. However, the levels of both cytokines in animals that treated with WPC or FSE alone were comparable to the control group. On the other hand, animals received tienilic acid and treated with WPC or FSE showed a significant improvement in TNFα level but insignificantly affect IL-6 level.

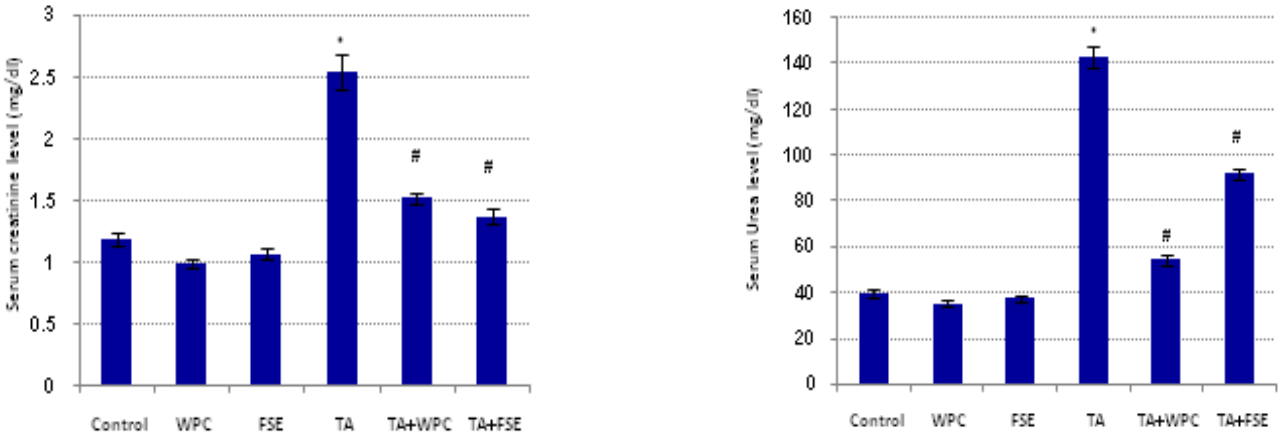

Figure 1. Serum creatinine and urea levels in rats treated with whey protein concentrate (WPC), fennel seeds methanolic extract (FSE), tienilic acid (TA), TA+WPC and TA+FSE. Data represent the mean ± standard error (n = 10). *p ≤ 0.05 compared to normal control rats; # p ≤ 0.05 compared to TA-treated rats.

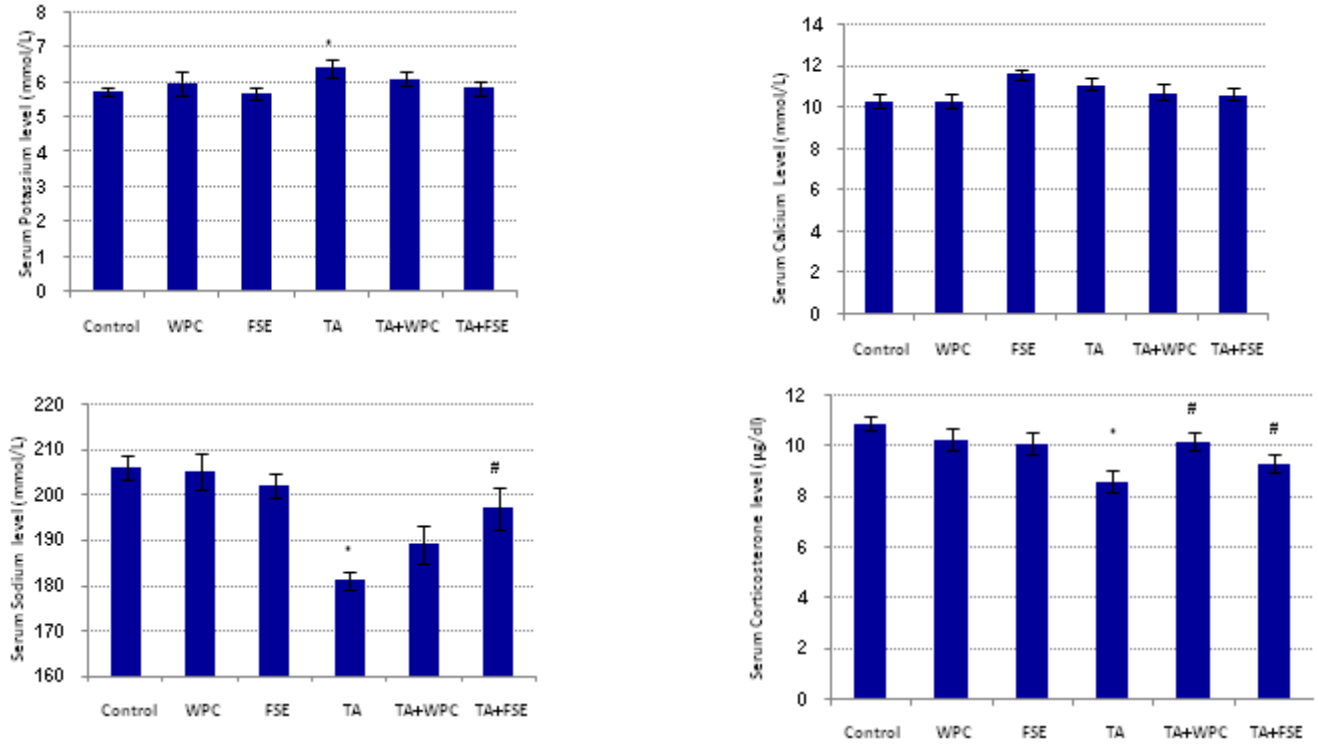

Figure 2. Serum potassium; sodium; calcium and corticosterone levels in rats treated with whey protein concentrate (WPC), fennel seeds methanolic extract (FSE), tienilic acid (TA), TA+WPC, and TA+FSE. Data represent the mean ± standard error (n = 10). * $p \leq 0.05$ compared to normal control rats; # $p \leq 0.05$ compared to TA -treated rats.

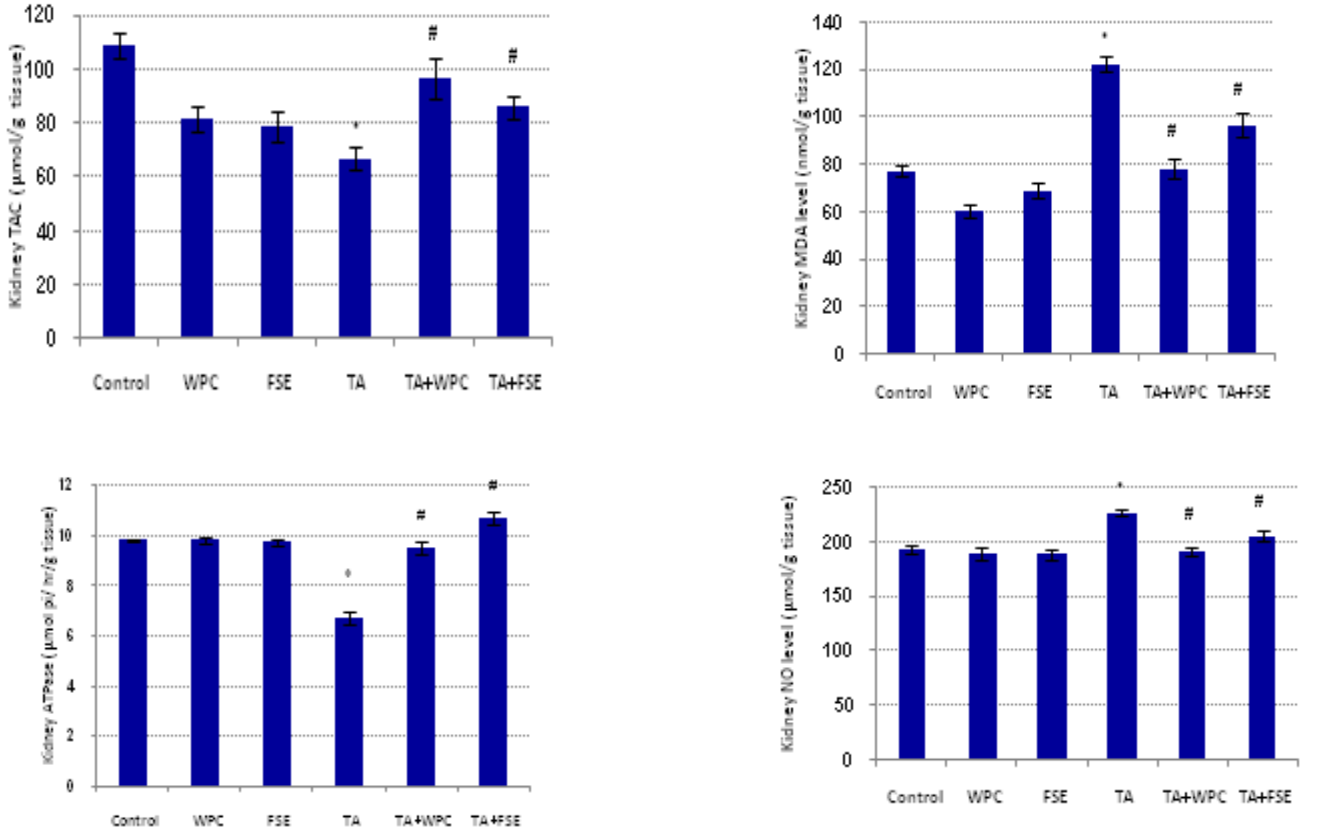

Figure 3. Kidney total antioxidant capacity (TAC); kidney Na^+/ K^+-ATPase activity (ATPase); kidney malondialdehyde (MDA) content; and serum nitric oxide level (NO) in rats treated with whey protein concentrate (WPC), fennel seeds methanolic standard error (n = 10). * $p \leq 0.05$ compared to normal control rats; # $p \leq 0.05$ compared to TA -treated rats.

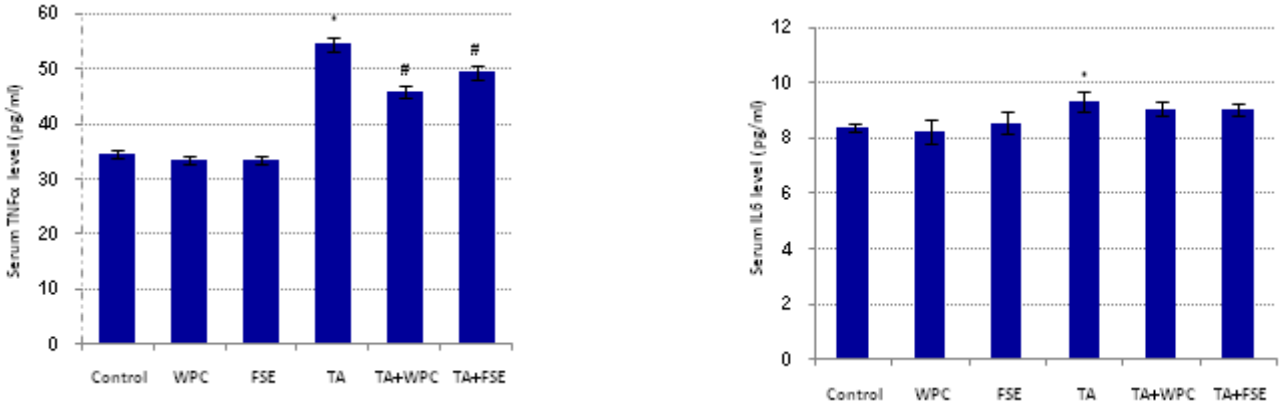

Figure 4. Serum tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) levels in rats treated with whey protein concentrate (WPC), fennel seeds methanolic extract (FSE), tienilic acid (TA), TA+WPC and TA+FSE. Data represent the mean ± standard error (*n* = 10). * $p \leq 0.05$ compared to normal control rats; # $p \leq 0.05$ compared to TA -treated rats.

Discussion

In the present study, we evaluated the protective effects of fennel methanolic extract (FSE) and whey protein concentrate (WPC) against the nephrotoxicity induced by tienilic acid administration in rats. In our study, treatment with tienilic acid resulted in a significant increase in urea and creatinine levels. These results may indicate impairment in kidney function. The measurements of serum urea [30] and creatinine [31] levels are considered to be good indicators for glomerular filtration rate. It was suggested that the reduction in glomerular filtration rate with an increase in serum blood urea nitrogen and creatinine levels indicates induction of acute renal failure [32]. A significant increase in plasma creatinine level was observed in patients with secondary hyperuricemia received tienilic acid [33]. Others found that tienilic acid treatments does not alter creatinine level [34]. This discrepancy is returned to the variation in the duration and dose of tienilic acid. Several reports linked renal failure in cases on tienilic acid treatments with urate deposition in the kidney [35-37]. Inadequate fluid intake and high starting doses of tienilic acid appear to be common to these cases [38].

The present data demonstrate significant decrease in plasma sodium and corticosterone levels concomitant with an increase in potassium level by tienilic acid treatment as compared to untreated control. Sodium (Na+) and potassium (K+) transport through the distal tubules is regulated by the action of adrenocortical hormones and aldosterone which promotes the increase in Na+ reabsorption in exchange for K+ [39]. Corticosterone, a mineralocorticoid, is one of the adrenocortical steroid hormones that regulate salt homeostasis (sodium conservation and potassium loss) and extracellular fluid volume [40, 41]. Mineralocorticoid deficiency leads to dehydration with hypotension, hyponatremia, and hypercalemia [42].

Tienilic acid may be suppress production of renin by the kidney and consequently decreases the plasma corticosterone level which in turn decreases the reabsorption of Na+ with concomitant decrease of K+ and H+ excretion. Therefore, plasma Na+ level was decreased while that of K+ was increased. In healthy kidney, receptors located in the juxtaglomerular cells when affected by decreased plasma Na+, they stimulate the production of renin enzyme. Renin is released into the blood, where it hydrolyzes its substrate angiotensinogen that is synthesized in the liver, to produce angiotensin I which is rapidly converted to angiotensin II by angiotensin- converting enzyme. Angiotensin II stimulate adrenal gland to produce mineralocorticoid hormones to maintain electrical neutrality [43].

Alternatively, It was reported by McLeod [44] that the antihypertensive nonselective β-adrenergic-receptor antagonists (β-blockers) are more effective than β-blockers in suppressing plasma renin activity when given in low doses and that they may thus be more appropriate for the treatment of hypertension in patients with elevated plasma renin activity.

The nephrotoxicity induced by tienilic acid may be a consequence of the increase in oxidative stresses. Oxidative stress *in vivo* can result from a reduction in endogenous antioxidant, burst formation of reactive oxygen species (ROS), or other imbalances between antioxidants and ROS. [45].

Tienilic acid is metabolized in the hepatic cells, where the thiophene ring of tienilic acid is oxidized by cytochrome P450 (CYP) 2C9 in humans, and similarly by CYP2C11 in rats [46,5,47] to form the labile electrophilic reactive intermediates, S-oxide and arene oxide, which are converted non-enzymatically to the major metabolite, 5-hydroxy tienilic acid (5-OHTA) [48]. 5-OHTA scavenged by intracellular reduced glutathione (GSH), or covalently

bound to macromolecules including metabolizing enzyme [49,50].

The covalently bound complex causes immune-mediated mechanisms that leads to toxicity, this asserted by the presence of the anti-liver and -kidney microsomal type 2 autoantibodies that specifically recognize CYP450 2C9 in humans [49] and the corresponding isoform 2C11 in rats [8].

In the present study, rats received tienilic acid showed a significant increase in the levels of renal malondialdehyde (MDA) and nitric oxide (NO) accompanied by a significant decrease in total antioxidant capacity (TAC) value. These results indicate the evolution of a state of oxidative stresses.

Nitric oxide serves beneficial roles as a messenger and host defense molecule but excessive NO production can be cytotoxic. The overproduction of NO leads to its reaction with reactive oxygen and form the most potent injurious peroxynitrite molecule [51]. Excessive NO production contribute to the pathogenesis of a number of renal diseases characterized by inflammation and injury such as tubulointestinal renal failure [52], glomerulo-nehritis [53] and postscemic renal failure [54].

The oxidative stress mainly results from endogenous antioxidant depletion due to the conjugation of GSH with tienilic acid metabolites. It was reported that GSH depletion leads to alteration of redox state in kidney and consequently leads to an increase in generation of superoxide and other oxygen radicals [55] that attach the lipids of cell membrane causing lipid peroxidation and increase MDA production.

TAC includes enzymes such as SOD, catalase and GPX and non-enzymatic antioxidants such as GSH and trace elements. TAC may provide more relevant biological information compared to that obtained by the measurement of individual components, as it considers the cumulative effect of all antioxidants present in plasma and body fluids [56]. The decrease in TAC and the increase in MDA reported herein in tienilic acid -treated rats leading to an indirect increase in oxidative DNA damage and in turn cell damage.

The current study revealed also a significant reduction in Na^+/ K^+-ATPase activity in tienilic acid - treated rats. Na^+/ K^+-ATPase is present in the plasma membranes of the cells and organelles. ATP degradation is an essential process for survival, and ATP is catalyzed by Na^+/ K^+-ATPase [57]. It concerned with the active transport of sodium-potassium across the cell membrane and responsible for a large part of the energy consumption constituting the cellular metabolic rate [58]. Moreover, it is highly dependent on lipids, therefore membrane lipid peroxidation affects the activity of this enzyme [59]; hence the oxidative stress induced by tienilic acid leads to lipid peroxidation that disturbs the lipid structure of membranes causing loss of Na+/ K+-ATPase activity.

Our study showed that tienilic acid increased the blood levels of IL-6 and TNF-α. IL-6 and TNF-α are proinflammatory cytokines involved in a variety of inflammatory responses, including differentiation, maturation and activation of inflammatory cells such as macrophages, neutrophils, T cells and natural killer cells [60]. The covalent binding of electrophilic metabolites of tienilic acid with proteins induces their structural modification, which may be recognized as a foreign antigen, leading to immune cells stimulation [61] and autoantibodies production. It is reported by Ramseyer and Garvin [62] that TNF-α alters renal hemodynamics and nephron transport, affecting both activity and expression of transporters. It also mediates organ damage by stimulating immune cell infiltration and cell death.

There is good evidence indicating that uraemia in general is associated with enhanced oxidative stress [63]. Accordingly, the impairment in kidney function reported in the present work in response to tienilic acid intake could be mediated by oxidative stress. Therefore, natural antioxidants have been proposed as therapeutic agents to protect against kidney damage.

Our data demonstrated that WPC and FSE are considered as potent antioxidant agents since they could produce marked decrease in MDA and NO formation that increased by the tienilic acid towards the normal values. We observed also that the lowered values of TAC and ATPase caused by tienilic acid tratment were effectively countered by WPC and FSE. Additionally, WPC and FSE could also ameliorate serum corticosterone and sodium but not affect calcium or potassium. These positive alterations were more pronounced in the animals received WPC.

WPC and FSE may increase the endogenous antioxidant capacity of the kidney to overcome oxidative stress induced by tienilic acid; this in turn improves kidney integrity and function and consequently enhance renal excretory function of urea and creatinine as well as enhances the kidney to maintain electrical neutrality and improve body homeostasis. In previous studies, it was found that WPC [22] and FSE [24] have free radical scavenging activity and in turn prevents the attack and exhaustion of the endogenous antioxidants. WPC and FSE contain a number of bioactive compounds which are generally believed to be the active constituents responsible for their antioxidant activity. In previous studies, we determined the major components responsible for the antioxidant activity of the tested materials that included thiol (SH) groups which was expressed as cysteine equivalents in WPC [22] and total phenolic compounds which was expressed as catechin equivalents in FSE [24].

The antioxidant activity of WPC may attributed to its higher content of cysteine and glutamate residues which are suggested that their intake contribute to increase the level of free cysteine, and consequent production of GSH

[64,65,21]. GSH, a tripeptide product synthesized from cysteine, glutamate, and glycine, is a low-molecular-weight thiol reductant present in most cells [66]. GSH was reported to be an antioxidant and anticarcinogenic, and thereby improving protection against oxidant-induced cell damage [67].

Whey protein contains also alpha-lactalbumin, lactoferrin, glycomacropeptide, beta-lactoglobulin and immuno-globulins which demonstrate immune-regulating properties [68]. The present data revealed that WPC administration counteracted the levels of blood IL6 and TNF-α that increased by tienilic acid. The study of Badr *et al.* [69] revealed that WPC could regulate levels of TNF, IL-1α, IL-1β and IL-10, thus decreasing inflammation. There is evidence suggesting that the development of diseases is associated with spontaneous increases in proinflammatory cytokines [70]; thus, the ability of WPC in decreasing these cytokines improves immune response and decreases the risk of the associated diseases.

The possible protective effects reported in this study are generally associated with the antioxidant activity of the polyphenolics. Polyphenols are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or aggression by pathogens [71]. Epidemiological studies strongly suggest that long term consumption of diets rich in plant polyphenolic compounds may help to prevent oxidative damage such as lipid peroxidation which is associated with cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases [72,73].

The methanolic extract of fennel seed was reported to increase the plasma superoxide dismutase and catalase activities and decreases lipid peroxidation in mice [74] and rats [13] models. The extract was reported to have also remarkable anti-inflammatory [75,13] and also anticancer properties [74].

Conclusion

In conclusion, the increase in the endogenous antioxidants and the reduction of proinflammatory cytokines and lipid peroxidation by WPC and FSE may result in reducing the deleterious effects due to the accumulation of free radicals in kidney tissue. The renoprotective effect of these agents might be through the prevention or scavenging of free radicals. This may be attributed to the high content of antioxidant compounds in WPC and fennel extract.

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