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Study the Possible Effect of Inula Graveolens L. Methanolic Extract on Antioxidant Status i n Track and Field Male Athletes

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Abstract

The antioxidant activity of *Inula graveolens* L. methanolic extract could be attributed to its higher content of both phenolic and flavonoid compounds. Therefore, the present study was undertaken to assessment the possible effect of methanolic extract of *Inula graveolens* L. plant as an improvement of enzymatic and non-enzymatic antioxidant status in track and field male athletes. Levels of enzymatic, non-enzymatic antioxidants and other general biomarkers were determined in serum and urine before (D_0) and after (D_{30}) supplementation of 400 mg of MEIG or placebo once at day for 30 day in elite group. This study showed increased significantly ($P < 0.01$) in the levels of vitamines A, E and C with compared to placebo group. On the other hand, no significant variation was seen in the levels of CAT, GRx, and SOD activities was disclosed; whereas, such was not the case as regards glutathione peroxidase (GPx). This enzyme activity decreased during the placebo period ($p < 0.05$) while remaining unaltered throughout the MEIG intake period. Same table reflect that the CK activity reached 686 ± 1.5 and 470 ± 1.4 (U/L) after placebo and MEIG periods, respectively ($P < 0.01$). Furthermore, data obtained, was shown that during placebo supplementation, urinary excretion of isoprostanes was markedly increased, disclosing thereby, an increase in oxidative injury and inflammation (1.29 ± 0.11 *vs* 1.75 ± 0.18 ng/mg creatinine at D_0 and D_{30}, respectively; $p < 0.05$). Such an increase did not appear during the MEIG supplementation (1.35 ± 0.12 *vs* 1.37 ± 0.13 ng/mg at D_0 and D_{30}, respectively). Finally, no significant difference on cholesterol and triglycerides was evidenced whatever the period (placebo or MEIG). Regardless of the capsule content the athletes had, no modification of urea concentration was observed; whereas, ferritin concentration was significantly reduced in both cases. Also, Hb concentration was not modified in the placebo group; however, MEIG supplementation increased Hb concentration, as opposed to placebo intake, respectively (12.8 ± 0.3 *vs* 12.6 ± 0.2 g/dL, $p < 0.05$).

Introduction

Reactive oxygen species (ROS) such as peroxynitrite, superoxide, and superoxide of hydrogen are constantly produced by cells that promote cellular oxidative damage. In normal conditions, mitochondrial aerobic metabolism is an important source of ROS production and these free radicals are neutralized by an elaborate antioxidant system including

superoxide dismutase, glutathione, peroxidase and catalase [1]. Additionally to enzymes, non-enzymes antioxidant agents play a key role to detoxify ROS from our body including vitamins A, E and C [2]. Erythrocytes are susceptible to oxidative damage as a result of the high polyunsaturated free fatty acid content of their membrane and the high cellular concentrations of oxygen and haemoglobin, a potentially powerful promoter of oxidative processes [3]. Erythrocytes are exposed to ROS that are constantly generated from both internal and external sources even under normal conditions, and they may be targeted for oxidative damage during exercise. However, erythrocytes, as well as the whole body, contain an elaborate antioxidant defence system that includes antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase and non-enzymatic anti-oxidants such as tocopherols, ascorbate, urate and glutathione (GSH). Alpha-tocopherol acts to protect polyunsaturated fatty acids in biological membranes against lipid peroxidation [4]. The effects of vitamin E supplementation on athletic performance and endurance [5] and lipid peroxidation [6] have already been investigated. However, the exercise studies have varied in the intensity, duration and mode of activity chosen for the study model. *Inula graveolens* L. is widely distributed in Mediterranean region and Middle East to West Pakistan. In Iraq (Basrah and lower Iraq), this plant is well known in Arabic and Eng- lish system as “Shuwaser, Suawaid” and “Strong-smel- lind *Inula*”, respectively [7]. Information gathered from some herbalists (in Basra governorate, Iraq) that the plant is useful to reduce rheumatic fever, infant convulsions, toothache, blood sugar, and also to dissolve internal blood clots, and to aid digestion [8]. Thus, the aim of the present work is to assessment the possible effect of methanolic extract of *Inula graveolens* L. plant as an improvement of enzymatic and non-enzymatic antioxidant status in track and field male athletes.

2. MATERIALS AND METHODS

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO), and solvents were from E. Merck (Darm- stadt, Germany). All of the reagents were prepared in deionized distilled water to eliminate the contamination of metal ions.

2.2. Plant Material and Extraction Procedure

Inula graveolens L. plant, used in this study, was collected on October 2016 from Abu-Al-Khaseeb region (Southern of Basra), Iraq. The plant was botanically authenticated and voucher specimens 3897 were deposited in the Herbarium of Basra (Iraq, Basra, College of Science, University of Basra). A published method by Al-Fartosy [8] was adopted. A quantity (100 g) of powdered plant was extracted in a Soxhelet apparatus with 80% methanol, for 24 h. The methanol extract was filtered and evaporated to

dryness under reduced pressure in a rotary evaporator to afford 9.47 g of dry extract.

2.3. Subjects

Male volunteer's track and field athletes were recruited after having submitted their written consent. They were informed of the purpose of this study and the possible risks involved before giving their oral consent to participate. Participants proved to be in good health as assessed by way of a medical history questionnaire, physical examination, and clinical laboratory tests. All subjects fulfilled the following eligibility criteria: 1) Practice of high level national intensive sports under non-stop training conditions over a one-month period, including competition; 2) no metabolic disorders (type 1 or 2 diabetes, cardiovascular, hepatic, gastrointestinal or renal diseases); 3) no pharmacological treatment, antibiotic nor supplemental vitamin and mineral use over the 8 weeks prior to launching our study; 4) fewer than 2 cigarettes per day; 5) under 20g per day alcohol intake; 6) no vegetarian, vegetalian nor deviated diet behavior; 7) no recent surgery; 8) no blood transfusion over the three months prior to the study; and 9) no involvement in another clinical study. Only 16 participants fulfilled the inclusion criteria and were included in this study. The volunteer's (±S.E.M.) age was 23.8±0.9 years, height 180±2 cm and weight 70.0±1.5 kg. The values were in the range of normal subjects with the same age. Their VO_2 max was 80.2±1.6 ml. kg^{-1}. min^{-1}, which was higher than that of normal subjects.

2.4. Study Design

The subjects, randomly divided into 2 groups, were assigned to supplementation either 400mg of MEIG or placebo (maltodextrin) under capsule forms (2 capsules) with their breakfast once at day for 30 day. The exercise was a mountain stage (3 km) daily for 30 day. All the volunteers performed the exercise protocol in the afternoon, and they were instructed to arrive at the laboratory in a rested and to avoid strenuous exercise 48 hours before the exercise protocol. After one month of supplementation and two weeks of wash out period, the treatments were reversed. Blood and urine samples were done at day 0 (D_0) and day 30 (D_{30}) of the period. The cyclists took a mean ±S.E.M. time of 9±0.5 min to complete this stage.

2.5. Sample Collection

Venous blood samples were taken from the antecubital vein after overnight fasting before and after exercise protocol. Rubber tourniquet was applied for less than one minute and the site to be punctured cleaned with 70% methylated spirit. A single blood sample was collected from each subject. Ten ml of blood was taken. About 1ml blood was placed into EDTA/K_3-coated tube to perform hemoglobin (Hb) analysis conducted less than two hours after sampling. The rest of the blood samples were placed in plain tubes and allowed to clot. After the blood had clotted it was placed in a

centrifuge and spun at 402 x g for 10 minutes to obtain the sera. The obtained sera immediately use in detection of variables in this study, and others were stored in deep freezing at (-20ºC) until using. Urine samples were collected in sterile tubes for creatinine and isoprostane analysis.

2.6. Measurements of serum enzymatic and non-enzymatic antioxidant

Superoxide dismutase activity in serum was determined by using a modified photochemical nitroblue tetrazolium (NBT) method utilizing sodium cyanide as peroxidase inhibitor [9]. Catalase (CAT) activity was measured by the decrease in absorbance due to H_2O_2 consumption (ε = 0.04 $mM^{-1}cm^{-1}$) [10]. Measurement of Glutathione reductase activity is based on the oxidation of NADPH to $NADP^+$ catalyzing by a concentration of glutathione reductase. One molecule of NADPH is consumed for each molecule of GS-SG reduced. Therefore, the reduction of GSSG is determined indirectly by the measurement of the consumption of NADPH at the wave length 340nm [11]. Glutathione peroxidase-1 activity was measured using an adaptation of the spectrophotometric method of Flohe´ and Gunzler [12]. This assay required H_2O_2 as a substrate and glutathione reductase and NADPH as an enzyme indicator. Glutathione peroxidase-2 activity was determined as for glutathione peroxidase-1 but the substrate was cumene hydroperoxide. Creatine kinase (CK) utilizes creatine phosphate as substrate to act as the initial catalyst for series of reactions resulting in the formation of NADPH as outlined in the coupled enzyme assay to CK activity and it is used to reduce nitroblue tetrazolium (NBT), in the presence of diaphorase, to give the blue, violet color of diformazan which has an absorption maximum around 560nm [13]. A modified fast accurate gas chromatographic method was described by Gawlik et al. [14]; and Zhao et al. [15] for detection of vitamin E (α-tocopherol) in serum. Vitamin A (Retinol) was detected with a modified method was described by Zaman et al. [16], while vitamin C (Ascorbic acid) was detected with a modified method described by Rumelin et al. [17] and Zhao et al. [15].

2.7. Measurements of Urinary isoprostanes:

The level of isoprostanes in urine samples was determined using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI and Oxford Biomedical Research, Oxford, MI). The amounts of isoprostanes were expressed in nanograms and corrected with creatinine values.

2.8. Measurements of General biomarkers: Triglycerides, Total cholesterol, ferritin, urea, and red blood cell hemoglobin

Serum triglycerides and cholesterol levels were determined using commercial kits (Biotrol, Paris, France and Biomérieux, Charbonnière-les-bains, France, respectively). Serum ferritin was assessed using a specific enzymatic kit (Roche Diagnosctics, Meylan, France). Serum urea was assessed using specific enzymatic and colorimetric kit methods (Biomérieux,

Craponne, France). Red blood cell Hb was measured using a haematology blood cell counter (Sysmex XT2000i, Roche Diagnostics, Meylan, France).

2.9. Statistical Analysis

The data were expressed as mean values ± SEM and tested with analysis of variance followed by Dunnett's t-test. P-values < 0.05, 0.01 were considered to be statistically significant.

Result and Discussion

In this work, supplementation of 400mg of MEIG once at day for 30 day lead to increased significantly (P **<** 0.01) in the levels of vitamines A, E and C with compared to placebo group (Table 1). On the other hand, no significant variation was seen in the levels of CAT, GRx, and SOD activities was disclosed; whereas, such was not the case as regards glutathione peroxidase (GPx). This enzyme activity decreased during the placebo period (p < 0.05) while remaining unaltered throughout the MEIG intake period. Same table reflect that the CK activity reached 686 **±** 1.5 and 470 **±** 1.4 (U/L) after placebo and MEIG periods, respectively (P **<** 0.01). Furthermore, data obtained in the Table 1, was shown that during placebo supplementation, urinary excretion of isoprostanes was markedly increased, disclosing thereby, an increase in oxidative injury and inflammation (1.29 **±** 0.11 *vs* 1.75 **±** 0.18 ng**/**mg creatinine at D_0 and D_{30}, respectively; p < 0.05). Such an increase did not appear during the MEIG supplementation (1.35 **±** 0.12 *vs* 1.37 **±** 0.13 ng**/**mg at D_0 and D_{30}, respectively). Finally, no significant difference on cholesterol and triglycerides was evidenced whatever the period (placebo or MEIG) (**Table 1**). Regardless of the capsule content the athletes had, no modification of urea concentration was observed; whereas, ferritin concentration was significantly reduced in both cases. Also, Hb concentration was not modified in the placebo group; however, MEIG supplementation increased Hb concentration, as opposed to placebo intake, respectively (12.8 **±** 0.3 *vs* 12.6 **±** 0.2 g**/**dL, p < 0.05; **Table 2**).

Table 1. Levels of enzymatic and non-enzymatic antioxidants before (D_0) and after (D_{30}) supplementation of MEIG or placebo in elite group.

	Placebo (n=8)		**MEIG (n=8)**		**P-value**
	D_0	**D_{30}**	**D_0**	**D_{30}**	
Levels of enzymatic antioxidants					
SOD (U/L)	27.1 ± 1.2	27.9 ± 1.4	27.6 ± 2.1	27.**8** ± 1.1	NS
GPx-1 (U/L)	69.6 ± 3.3	65.3 ± 2.1	68.2 ± 1.3	66.9 ± 1.3	NS
GPx -2(U/L)	28.3 ± 1.8	25.3 ± 1.5	27.**8** ± 1.1	26.9 ± 1.4	NS
CAT (K/ml)	40.3 ± 2.2	38.4 ± 1.3	40.1 ± 1.4	42.8 ± 1.2	NS
GR_X (U/L)	26.6 ±	25.9 ±	28.1 ±	26.6 ±	NS

	1.4	3.1	**1**.2	**1**.3	
CK (U/L)	519 ± 1.1	686 ± 1.5	595 ± 1.3	470 ± 1.4	HS
Levels of non-enzymatic antioxidants					
Vit E (µg/mL)	12.39 ± 0.13	12.40 ± 0.24	12.01 ± 0.19	13.88 ± 0.61	HS
Vit E/Tc ratio (µg/mg)	6.96 ± **0**.03	7.20 ± 0.00	6.86 ± 0.04	7.88 ± 0.02	HS
Vit C (µmol·L-1)	60.6 ± 2.8	55.5 ± 2.4	56.8 ± 1.1	62.5 ± 2.3	HS
Vit A (µmol·L-1)					HS
Isoprostanes (ng/mg creatinine)	1.29 ± 0.11	1.75 ± 0.18	1.35 ± 0.12	1.37 ± 0.13	S

Table 2. Levels of Hemoglobin and general serum biomarkers before (D_0) and after (D_{30}) supplementation of MEIG or placebo in elite group.

	Placebo (n=8)		**MEIG (n=8)**		**P-value**
	D_0	**D_{30}**	**D_0**	**D_{30}**	
Tg (g/ L)	0.63 ± 0.03	0.67 ± 0.04	0.69 ± 0.05	0.79 ± 0.08	NS
Tc (g/ L)	1.78 ± 0.02	1.72 ± 0.03	1.75 ± 0.05	1.76 ± 0.02	NS
Ferritin (µg/ L)	74.7 ± 7.6	67.1 ± 7.2	76.8 ± 8.7	67.6 ± 8.86	HS
Urea (g/ L)	0.276 ± 0.01	0.268 ± 0.03	0.291 ± 0.02	0.281 ± 0.06	NS
Hb (µg/ dL)	12.8 ± 0.4	12.5 ± 0.3	12.6 ± 0.2	12.8 ± 0.3	NS

In the present study, I hypothesized that a methanolic extract of *Inula graveolens* L. plant (MEIG) supplementation at physiological doses would partially avoid antioxidant system down-regulation and consequently lower chronic and/or acute exercise-induced oxidative damage in elite athletes while in competition. Moreover, oxidative stress generated under such conditions is likely to trigger oxidative skeletal muscle fatigue and damage [18] which can affect exercise performance. Thus, the second objective of this study was to cross-evaluate the effect of the MEIG supplementation on these parameters, along with oxidative stress and the antioxidant status. The antioxidant activity of *Inula graveolens* L. methanolic extract could be attributed to its higher content of both phenolic and flavonoid compounds [8]. Therefore, the ingestion of 400mg of MEIG per day during one month produced a real increase of vitamin A, vitamin E and C compared to placebo ingestion. However, antioxidant vitamin levels of all sportsmen participating in the study were within the normal range of well-nourished people [19]. The difference between the vitamin E level in the supplemented group and in the placebo is lower than others described in similar experiences with well-nourished adults [20] and similar to others with sportsmen [21]. Probably sportsmen should take a higher amount of vitamin E than normal adults in order to protect themselves against the oxidative stress associated to physical activity [22]. The liposoluble nature of these compounds A and E are responsible for the body accumulation. On the other hand, the increase

of plasma ascorbate levels in the MEIG-supplemented group was the lowest of the ones produced by the extract supplementation. The hydrosoluble nature of this vitamin and the existence of homeostatic mechanisms to regulate ascorbate plasma levels [23] could be responsible for this low plasma increase. It is difficult to raise blood levels of vitamin C beyond a certain value and they could be attained within 15 days of diet supplementation [24]. These results are similar to others described in the literature [25] and represent a real increase of the sportsmen cellular antioxidant availability. These results evidenced that MEIG consumption increases the athletes' antioxidant status; they further revealed, however, that the interpretation of the changes in plasma antioxidant capacity relies for the most part on the method used, a case in point formerly demonstrated by Prior and Cao [26]. The increase of the plasma vitamin E concentration and the preservation of the GPx levels could be explained by the increase of the Oxygen radical absorbance capacity value and the limitation of the Ferric reducing ability of plasma value reduction induced by the consumption of MEIG phenolic compounds. The same result on vitamin E was shown in endurance athletes [27] or in triathletes [28] after absorption of different antioxidant blends. Palazzetti et al. [28] also demonstrated the significant increase of GPx activity after antioxidant supplementation compared to placebo. As regards amateur male athletes while under competition conditions, the antioxidant supplementation maintained the glutathione reductase activity in the treated group after three months; whereas, it decreased significantly in the placebo group [29]. This decrease was also exhibited in soccer players having volunteered to participate in an incremental treadmill running exercise [30]. This enzyme is clearly linked with GPx because it regenerates the oxidized glutathione, cofactor of GPx; it is noteworthy therefore, that there was solid evidence of enzyme activity preservation, akin to our GPx related findings. Even if the type and the dose of antioxidant supplementation differ in these studies, results are congruent with the present study. Other blood antioxidant determined biomarkers, enzymatic such as superoxide dismutase, catalase or non enzymatic such as vitamin C, did not differ significantly whatever the period. These results are in agreement with those of Palazzetti et al. [28] and Margaritis et al. [31]; the latter revealed no variation in vitamin C and SOD in supplemented triathletes compared to placebo. Concerning catalase activity, Tauler et al. [32] studied its variation on athletes supplemented with vitamin C, or with a blend of vitamin E, vitamin C and beta-carotene [29]. In both cases they exhibited an increase in catalase activity that was not disclosed in our study. One of the biomarkers most commonly investigated within biological systems remains the degree of lipid peroxidation; to such an end, several methods may be implemented [33]. In the present study, lipid peroxidation was

determined in urine using the ELISA method to determine isoprostanes. Urinary isoprostane values were significantly increased in the placebo group, but were not modified in the MEIG group. Therefore, compared to the placebo, MEIG limited the production of isoprostanes significantly after the administration period. Isoprostanes are specific end-products of non enzymatic free radical catalyzed oxidation of arachidonic acid. It is believed that isoprostanes are formed while still esterified to phospholipids in cellular membranes and are released through the action of phospholipase to circulate freely in the body [34,35]. Quantification of isoprostanes has been referred to as the ultimate criterion for *in vivo* lipid peroxidation and oxidative stress, notably in acute or chronic inflammatory conditions [36]. An increase in isoprostane concentration has been reported in athletes during exercise in several studies [37], showing induced-exercise oxidative stress. The effect of antioxidant supplementation on this biomarker has been probed and widely documented as regards smokers, menopausal women or patients with degenerative diseases [38,39,40,41]. The effect on athletes, however, remains subject for debate [42,43]. The present trial is the first one ever to substantiate evidence that a MEIG supplementation during one month optimizes significant limitation of exercise-induced urinary isoprostanes production. This result is probably explained by the significant increases of vitamin E concentration, of GPx activity and by the presence of flavanol metabolites in the body. Numerous studies have reported that exogenous antioxidants protect against skeletal muscle fatigue. Also, these studies, however, have applied synthetic or enzymatic antioxidants and resorted to *in vitro* muscle preparations to quantify muscular contractions [44,45,46,47,48,49,50]. The determination of serum creatine kinase (CK) is used as an indirect index of exercise induced muscle damage [51] and is associated indirectly with the increment of the permeability in the muscle cell membrane [52]. This permeability is produced by the oxidative stress induced by physical exercise [53,54,55]. Morillas-Ruiz et al. [56] showed that consumption of a natural antioxidant beverage containing 1.2g per liter of polyphenols by cyclists increased the serum CK concentration similarly to the placebo beverage suggesting that the antioxidant capacity of polyphenols has no repercussion on cellular muscle damage. The same result was obtained by Helgheim et al. [57] with a vitamin E supplementation and by Margaritis et al. [31], on triathletes supplemented with an antioxidant blend. Contrariwise, while using the same sportsmen and supplementation the latter revealed a significant reduction of the magnitude in duathlon-induced creatine kinase isoenzyme mass increase during normal and overload training [28]. Branched-chain amino acids [58], vitamin C [59], allicin [60], or CoQ_{10} [61] supplementations on different volunteers generated the same result: a lower CK activity as compared to the control group.

Because of the supplement antioxidant effects, muscle membrane integrity should be maintained, preventing the release of enzymes into blood circulation. All these results are in agreement with the findings presented here, hereby exhibiting a trend towards decreasing the serum CK concentration among elite athletes involved in competition while absorbing the grape extract, as compared to placebo intake. Such a trend ($p = 0.09$) results from a very high variability already reported by Hartman and Mester [62]: authors observed that athletes with chronically low CK exhibited mainly low variability; those with chronically higher values exhibited considerable variability. Nevertheless, this is the first time that this modification has been reported under MEIG supplementation. Urea, ferritin and Hb markers are commonly used to determine overtraining signs [62,63]. No modifications appeared in urea concentration, one nitric substance considered as a marker of protein catabolism. Differently, ferritin concentrations are significantly decreased whatever the period. This decrease of ferritin concentration is well-defined during training associated with the competition season or overtraining [63,64,65]. Althogh, data obtained revealed that there was no difference in ferritin concentration between the placebo and the MEIG supplementations. Aguilo et al. [64] showed that supplementation with vitamins E, C and β-carotene (source of vitamin A) prevented the decrease of serum iron and the iron saturation index. Such a difference in results may result from the type of supplementation, the dose and/or the used model: amateur trained male athletes supplemented with 1.530g per day of pure vitamins for Aguilo and co-workers or elite trained male athletes supplemented with 400mg per day of a complex botanical extract for the present study. Several authors have described a significant increase in the destruction of the red blood cells after intensive exercise [66]. One of the causes for this hemolysis is the fact that after strenuous exercise, the red blood cells are more responsive to stress, such as oxidative stress [67]. Hb represents more than 97% of the dry content in the red blood cells, being the normal concentration in sedentary men between 13.5 and 16.5 g**/**dL [68]. In the present study, Hb was increased by 0.3 g**/**dL among elite athletes after MEIG administration. Other studies on sportsmen and antioxidant supplementation yielded no variation of this parameter [56,64]. Our results might suggest that the increase of blood antioxidant capacity, induced by MEIG, protected red blood cells against free radicals generated under competition conditions, but further research is needed in order to confirm this hypothesis.

Conclusion

This study suggested that the methanolic extract of *Inula graveolens* L. (MEIG) possesses antioxidant activity that might be helpful in ameliorate the oxidative stress/antioxidant status balance in elite athletes during a

competition period. Further investigation on the isolation and identification of antioxidant components in the plant may lead to confirm the link between the oxidative stress/antioxidant status balance, cellular protective action, and performance enhancement effects caused by the consumption of MEIG in elite and occasional athletes.

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