

Olive phenolics increase glutathione levels in healthy volunteers

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Running Title: Olive phenolics increase glutathione in humans

Abstract

Several in vitro and in vivo studies have shown that olive phenols exert potent
20 biological activities including, but not limited to, antioxidant actions. These activities
are shared by phenols found in olives, olive oil, and olive mill waste water (OMWW).
The aim of this study was to investigate whether a commercially available OMWW
preparation could influence some parameters of oxidative status in healthy human
volunteers. Ninety-eight healthy subjects with normal body weight were recruited and
25 5 ml of blood was drawn from their antecubital vein after an overnight fast of at least
12 h. After this, subjects were asked to ingest 2 ml of a commercially-available
OMWW preparation. Another 5 ml of blood were drawn one hour after the ingestion
of the preparation. Plasma antioxidant capacity and total and reduced glutathione
were measured. We did not observe any difference in plasma antioxidant capacity
30 between baseline and after one hour after the ingestion of the extract. Conversely, a
significant increase in total plasma glutathione concentration was measured. This
increase involved both the reduced and oxidized forms of glutathione; hence, their
ratio was unaffected by the treatment. The observed effects of OMWW on glutathione
levels might be governed by the antioxidant response element (ARE)-mediated
35 increase in Phase II enzyme expression, including that of γ -glutamylcysteine ligase
and glutathione synthetase. Future studies on groups of individuals who may benefit
from an increase in their glutathione levels, eg the elderly, will further elucidate the
biological activities of this formulation.

40 **KEYWORDS** : polyphenols ; hydroxytyrosol ; nutraceuticals ; olives ; natural
antioxidants; olive mill waste water.

INTRODUCTION

Olives contain high amounts of a variety of phenolic compounds, the most
45 abundant being the secoiridoid oleuropein, which is a combination of elenolic acid
and hydroxytyrosol (1). Several in vitro and in vivo studies have shown how both
oleuropein and hydroxytyrosol exert potent biological activities including, but not
limited to, antioxidant actions (2, 3). Indeed, interest in the healthful properties of
olives and olive oil, mostly due to their phenolic components, has increased recently
50 and has equaled that of other food items such as red wine and tea. During olive oil
production, according to their partition coefficient, olive phenolics end up either in
olive oil, in the olive mill waste water (OMWW), or in the residual, solid phase
(pomace) (4, 5).

In addition to the extensive research on extra virgin, i.e. phenol-rich olive oil,
55 data are also accumulating on the bioactive potential of OMWW preparations (6, 7).
Results from both in vitro and in vivo (including rats and humans) studies suggest
that OMWW, of which hydroxytyrosol is the most bioactive component, possesses
anti-inflammatory, anti-thrombotic as well as antioxidant properties and as such is
potentially capable of preventing passive smoke-induced oxidative stress, reduce of
60 thromboxane B₂ production by whole blood, and ameliorate symptoms of
inflammatory disease such as osteoarthritis (4, 5, 8-14).

The aim of this study was to investigate whether a commercially available
OMWW preparation could influence some parameters of oxidative status in healthy
human volunteers.

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MATERIALS AND METHODS

Design. The OMWW preparation we tested contained, as evaluated by HPLC coupled with UV detection, $\leq 6\%$ of simple and complex (including secoiridoids) phenols, of which 45% was hydroxytyrosol and 7.5% was oleuropein (Table 1).

70 Ninety-eight apparently healthy subjects with normal body weight were recruited from within the Prince Court Medical Center (PCMC, a private hospital located in Kuala Lumpur, Malaysia which is affiliated with the Medical University of Vienna) and in the city of Kuala Lumpur via word-of-mouth. The study protocol was approved by the local Ethic's committee, was fully explained to the participants, and
75 written informed consent was obtained by all subjects prior to starting the trial. Subjects were instructed not to eat or drink tea phenol-rich foods or beverages such as coffee, red wine, or chocolate the night prior to the experiment.

Experimental Procedure. For the assessment of the oxidative status of the study
80 population, at baseline, 5 ml of blood was drawn from the antecubital vein after an overnight fast of at least 12 h. After this subjects were asked to ingest 2 ml of a commercially-available OMWW preparation (Olivenol Livin'), diluted in a glass of water. This amount provided ~ 50 mg of hydroxytyrosol and ~ 8 mg of oleuropein. This dose was chosen based on previous studies of hydroxytyrosol's bioactivity (9, 12)
85 and is this commercial preparation's prescribed amount. Another 5 ml of blood were drawn exactly one hour after the ingestion of the preparation. Plasma was separated by centrifugation at $2100 \times g$ for 20 min at 4°C , aliquoted, and stored at -80°C .

 Plasma antioxidant capacity was evaluated utilizing the TAS kit (Randox, Crumlin, UK) (15), according to the manufacturer's instructions. Total and reduced
90 (GSH) glutathione were measured, after deproteinization, colorimetrically, following the manufacturer's (Cayman Chemical Company, Ann Arbor, USA) instructions (16).

RESULTS

We did not observe any difference in plasma antioxidant capacity between
95 baseline and after one hour after the ingestion of the extract (Table 2). Conversely, a
significant increase in total plasma glutathione concentration was measured. This
increase involved both the reduced and oxidized forms of glutathione; hence, their
ratio was unaffected by the treatment (Table 2).

100 DISCUSSION

This is the first study to report that a commercially available OMWW
preparation is capable to increase total plasma glutathione when administered to
healthy volunteers of various Asian ethnic descent. This increase, however, did not
translate into an increase in total antioxidant capacity in the studied population.

105 To counteract the potentially noxious effects of xenobiotics, higher vertebrates,
including humans, have developed a battery of genes encoding phase II and
antioxidant enzyme expression (17). These include the expression of various
superoxide dismutase (SOD) isoforms, catalase, glutathione peroxidase, glutathione
reductase, various glutathione-S-transferase (GST) isoforms, NAD(P)H: quinone
110 oxidoreductase 1 (NQO1), and heme oxygenase (HO)-1, which can exert
cytoprotective, antioxidant, and anti-inflammatory effects. One mechanism by which
cells respond to oxidative injury is through the antioxidant response element (ARE), a
cis-acting enhancer sequence that regulates the transcription of various
cytoprotective genes (18). Upon toxic injury, the transcription factor nuclear factor
115 erythroid 2-related factor 2 (Nrf2) translocates to the nucleus and dimerizes with
small Maf proteins to form a transactivation complex that, consequently, binds to the

ARE. (see (19) for details on Nrf2 regulation). Nrf2-induced ARE activation coordinates the expression of many genes involved in combating oxidative stress and toxicity in a broad range of tissues and cell types.

120 The synthesis of GSH from its constituent amino acids involves the actions of two ATP-dependent enzymes, γ -glutamylcysteine ligase (GCL) and GSH synthetase. GCL, the rate-controlling enzyme in the overall pathway, is a heterodimer composed of a catalytic (GCLC) and a modulatory (GCLM) subunit. The basal and inducible expressions of these GCL substituents are mediated by means of the ARE (20).

125 Pharmacological agents and natural substances shown to induce ARE-regulated gene expression (and, hence, are endowed with chemopreventive activities) include oltipraz, anethole dithiolethione (ADT), sulforaphane, 6-ethylsulphinylnhexyl isothiocyanate (6-HITC), curcumin, caffeic acid phenethyl ester (CAPE), as well as 4'-bromoflavone (18). In synthesis, the observed effects of
130 OMWW on glutathione levels might be governed by the ARE-mediated increase in Phase II enzyme, namely GCL and GSH synthetase, expression, in turn leading to enhance glutathione synthesis. If confirmed, the potential applications of these preliminary findings are manifold and could extend to patients with reduced circulating glutathione levels, for example patients on chronic hemodialysis (21, 22),
135 patients suffering from Alzheimer (23) or HIV-infection (24). Furthermore, elderly people may potentially benefit from an increase of glutathione levels (20). It must be highlighted though, that the orally ingested OMWW preparation investigated in the current trial acted solely on total glutathione levels and not on its reduced/oxidized ratio. Therefore, future investigations are crucial, in the context of a repeated
140 administration of OMWW preparation as well as dose finding studies, in order to evaluate a potential influence of OMWW on the reduced/oxidized ratio of glutathione.

The lack of effect on total antioxidant capacity observed in this study – which is in agreement with Kendall *et al.* (25) and with Schaffer *et al.* (13) - might be interpreted in terms of lower attainable concentrations of OMWW phenolics as compared to other endogenous antioxidants (26). Despite the paucity of data, it appears that OMWW phenolics, namely hydroxytyrosol, attain plasma concentrations in the low μM range (27). In addition, OMWW phenols such as hydroxytyrosol are extensively metabolized in humans (28) and data on the biological effects of metabolites are still scant. However, it is noteworthy that, as far as hydroxytyrosol is concerned, its 3-O-glucuronide conjugate exhibits strong antioxidant activities (stronger than those of the parent molecule) *in vitro* (29). Although these factors limit the capability of OMWW components to act as antioxidants in plasma *in vivo*, especially compared to other antioxidants present in plasma at high steady-state concentrations, e.g., 30–150 μM ascorbate (vitamin C), 160–450 μM urate, or 15–40 μM α -tocopherol (vitamin E) (26). Hence, it is possible that prolonged consumption of bioactive molecules such as olive phenols, might exert long-term beneficial health effects, as emphasized by studies where biological activities of OMWW preparations have been demonstrated in humans (9, 10, 12).

Concerns of OMWW toxicity have been extensively addressed by D'Angelo *et al.* (30), by Christian *et al.* (31), and by Soni *et al.* (32). These groups could not demonstrate toxic effects of hydroxytyrosol even at much higher doses as administered in this trial; hydroxytyrosol-rich OMWW extract has been granted GRAS status by the Food and Drug Association (FDA).

One limitation of our study was the lack of placebo. However, given the high number of subjects at study and the high statistical significance of our results, it is unlikely that the observed differences were due to chance. Another limitation was that

we did not evaluate hydroxytyrosol's bioavailability. However, this issue has been already fully elucidated by us and other investigators [e.g. (27, 28, 33, 34)].

In conclusion, the ingestion of 2 ml of a commercially available OMWW preparation has shown to be able to significantly increase total glutathione levels in 98 healthy volunteers, while not affecting plasma antioxidant capacity. Among the numerous groups of individuals who may benefit from an increase in their glutathione levels, the elderly might be paid special attention, considering the fact that overall life expectancy is constantly increasing. Future studies - including the evaluation of various dosages and long-term administration of OMWW - are warranted to further investigate this formulation.

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Table 1 Composition of the OMWW preparation at study.

Protein:	5-10%
Carbohydrates:	45-68%
Fat:	17-30%
Ash (%wt/wt)	8-15%
Lead:	< 2 ppm
Heavy metals:	< 10 ppm
Simple & Polyphenols:	6% (minimum)
• Hydroxytyrosol	40-50% of total phenolics
• Gallic acid	2-5% of total phenolics
• Tyrosol	2-5% of total phenolics
• Oleuropein	5-10% of total phenolics
• Other secoiridoids	10-20% of total phenolics

285 **Table 2** Plasma antioxidant and glutathione status of healthy volunteers before and after the administration of an OMWW preparation.

	Before	After
TAS (mmol/L)	2.067±1.363	1.996±1.291
GSH (μM)	1.794±1.471	2.837±1.617*
GSSG (μM)	0.403±0.485	0.741±0.620*
GSH/GSSG	13.55±21.49	10.86±18.47

Abbreviations: TAS, total antioxidant status); GSH, reduced glutathione; GSSG, oxidized glutathione. Data are means ± SD. *p< 0.01, Student's *t* test.