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Full Length Research Paper

Validation of neuroprotective action of a commercially available formulation of olive polyphenols in a zebra-fish model vis-a-vis pure hydroxytyrosol.

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The goals of this study were to assess and compare the effects of a commercially available formulation of olive polyphenols (HIDROX® 12% by Creagri, Inc. of Hayward) on cuprizone-induced zebrafish lethality and brain degeneration, and to compare its effects to those of pure hydroxytyrosol on cuprizone-induced brain degeneration. We chose the cuprizone model in light of its close association with neuroinflammation and neurodegeneration, and because the same model is an accepted model for the outcome of several neurodegenerative diseases, including multiple sclerosis, schizophrenia, epilepsy,Parkinson's and Alzheimer's.The comparison with pure hydroxytyrosol was directed to establish pure hydroxytyrosol (HT) which exerts a neuroprotective action comparable to the commercially available formulation of olive polyphenols tested and a meaningful difference in neuroprotection existed between the commercially available formulation and the purified hydroxytyrosol.

Key words: Olea europaea, polyphenols, hydroxytyrosol, neuroprotection, Hidrox®, Alzheimer's, Mediterranean Diet

INTRODUCTION

The beneficial effects of the Mediterranean diet on health and longevity have been recognized in literature by many researchers.(Altomare et al., 2013). The cardio-protective and anti-apoptosis effects of such diet based on fresh foods, vegetables, nuts, fish and healthy fats are now advanced by the medical community and presented as basic component of a healthy living lifestyle to people around the world. (Sofi et al., 2010).In this direction in 2006 the Annals of Internal medicine published the results of the Eurolive Study, a research conducted by the Institute Municipal d'Investigacions Medica (IMM). (Covas et al., 2016).In that instance, investigators found that olive oil's protective attributes against oxidative stress associated diseases, such as cardiovascular diseases, cancer, and neurodegenerative disease, besides to its high content of monounsaturated fatty acids, must also be credited to the antioxidant properties of olive polyphenols. A class of plant-based biogenic substances (which also includes flavonoids, carotenoids, and terpenoids), olive polyphenols are derived from fruit and the leaves of the Olea europaea (the olive tree). Several studies have credited them with having healthy and restorative properties, and for being responsible for the many positive outcomes of the Mediterranean diet, including cardioprotection and neuroprotection. The PREDIMED Trial (Prevención con Dieta Mediterrànea)—

a five-year multi-center, multi-year, randomized study involving 7774 subjects confirms this finding.(Estruch., 2013) Published in 2013 has revealed that about 30 percent of heart attack, strokes, and death from heart disease can be prevented if people at high risk just switched to a Mediterranean-like diet rich in olive oil, fresh vegetables,nuts, fish, poultry and other traditional foods from the Mediterranean basin.(Ibid.)The finding was so startling and definitive that the study was concluded ahead of schedule, since the results were so indicative of benefits that continuing it would have been unethical. (Guarneri., 2017) But although these molecules are known since Ancel Keys' discovery that the poor men of the Neapolitan harbor in Italy enjoyed a much better health than the wealthy bankers of Manhattan (The 7 Years Study, 2016), only recently researchers have focused on this diet to account for the region's low incidence of neurodegenerative disease and dementia in the aging population of the region. These findings have been successively found to be true also for the prevention of cognitive impairment.(Tort,2010)Oxidative stress and vascular impairment are in fact believed to be partly responsible for the cognitive decline in late life of the aging population, a strong predictive factor for the onset of senile dementia. (Justin et al, 2013) Epidemiologic studies suggest that adoption of a Mediterranean diet-like, an antioxidant-rich cardioprotective dietary pattern of nutrition, may also provide the restoration of a meaningful grade of cognitive function. (Wade et al, 2107).Although the Mediterranean diet combines several foods, micronutrients, and macronutrients, one molecule, in particular, stands out for its medicinal properties, 3,4 dihydroxyphenylethanol or hydroxytyrosol (HT).(Baldioli et al, 1996).A bio-phenolic molecule found almost exclusively in the Olea europaea; hydroxytyrosol is a phenylethanoid, a kind of phytochemical showing in-vitro antioxidant activity. Abundant in nature in the leaves and the fruit of Olea europaea in nature hydroxytyrosol presents in its elenolic acid ester form oleuropein and, especially after degradation, in its unbound form. (World Alzheimer Report 2015) Hydroxytyrosol has been investigated for decades for its antioxidant and cardioprotective activities; however, recent interest in the molecule has centered on its anti-inflammatory, (Przedborski et al, 2003) antioxidant (Bredesen, 2003) and brain protecting,(Przedborski et al, 2003, (Brambilla 2003) and mitochondria enhancing activities, while contributing to restoring healthy immune response. (Visioli. et al, 2000) However scientific literature is still inconclusive to what accounts precisely for the compound's broad spectrum cytoprotective and antioxidant activity. With some studies pointing to the grade of purity of the compound as marker activity (Fki et al, 2005) while some other seem to indicate that marker into the interaction between the minor components deriving from the metabolization of oleuropein and hydroxytyrosol in an enriched presence.(Oneda et al, 2003) Peer-reviewed studies in health and prevention published by independent researchers over the past years with Hidrox, and with consumer products containing Hidrox, indicated to a different rate of activity between the proprietary formulation and that of pure (or purified) Hydroxytyrosol. In a bovine heart endothelial cell-based assay, researchers at the University of Tokyo and the Tokyo Vascular Disease Institute found that while Hidrox® was able to prevent peroxidative damage cause by agents such as 15-HPETE, pure HT obtained by HPLC separation was not protective against oxidation. Furthermore in high concentrations of purified HT produced a pro-oxidation effect and enhanced the cytotoxicity of the oxidants.(Ibid.))In another study on the oxidation of mitochondrial membrane lipids by free radical by Electron Spin Resonance (ESR) spectroscopy (using Superoxide, HO radical and NO radicals as toxic agents), in vitro, researchers confirmed that Hidrox® had higher suppressing activity than the same amount of purehydroxytyrosol (HT) (Richard et al.,2011) Furthermore in 2011 scientists at Basel-based DSM Nutritional found that Hidrox® has a different activity profile than purified HT. Published in Planta Medica in 2011, the DSM scientists confirmed the power fulanti-inflammatory activity of Hidrox® and speculated that it must contain additional micro-components that contribute to its superior antioxidant activity. (Rodder., 2011)

MATERIALS AND METHODS

Compounds:

For the lethality study, master stock solution (MS) of HIDROX® 12%(total polyphenols; HT≥3%) (10 mg/ml) in dimethyl sulfoxide (DMSO), were provided by CreAgri. For the study to assess protective effects of HIDROX® and hydroxytyrosol on cuprizone-induced brain degeneration, five tubes of HIDROX® powder (1.5 g/tube) and hydroxytyrosol (5 five mg/ml) in DMSO were provided by CreAgri.

Standard procedures for zebrafish breeding:

Phylonix wild-type AB zebrafish were generated by natural mating, as described in the Zebrafish Handbook. Embryos were cleaned (dead embryos removal) and sorted by developmental stage. Because embryos receive nourishment from an attached yolk sac, they need no feeding for six days post fertilization (dpf).

Lethality test:

Treatment with 750 micromolar (μM) cuprizone for 42 hr was used to induce lethality in 2 days post fertilization (dpf) zebrafish. Final test conditions were: 1) untreated,

Table 1. Final conditions for lethality test

Condition	Final concentration
1) Untreated	0
2) Vehicle	0
3) HIDROX	100 µg/ml
4) Cuprizone	750 µM
5) HIDROX + cuprizone	100 µg/ml + 750 µM

Table 2. Final conditions for brain degeneration test

Condition	Final concentration
1) 0.4% DMSO	0
2) Vehicle	0
3) Cuprizone	1000 µM
4) HIDROX + cuprizone	400 µg/ml + 1000 µM
5) Hydroxytyrosol + cuprizone	80 µg/ml + 1000 µM

Table 3. Lethality test results (N =30)

	% Lethality				
Condition	**Exp 1**	**Exp 2**	**Exp 3**	**Mean**	**SD**
1) Untreated	0 (0/30)	0 (0/30)	0 (0/30)	0 (0/30)	0 (0/30)
2) Vehicle	0 (0/30)	0 (0/30)	0 (0/30)	0 (0/30)	0 (0/30)
3) HIDROX (100 µg/ml)	0 (0/30)	0 (0/30)	0 (0/30)	0 (0/30)	0 (0/30)
4) Cuprizone (750 µM)	70 (21/30)	67 (20/30)	70 (21/30)	69 (21/30)	2 (1/30)
5) HIDROX +cuprizone	13 (4/30)	30 (9/30)	43 (13/30)	29 (9/30)	15 (5/30)

Numbers in parentheses: number of dead zebrafish divided by number of animals per well.

2) vehicle (fish water containing 0.1 % dimethyl sulfoxide(DMSO) and 1% acetic acid, pH adjusted to 7.2), 3) HIDROX® (100 µg/ml) for 48 hr, 4) cuprizone (750 µM) for 42 hr, and 5) pre-treatment with HIDROX® (100 µg/ml) for 6 hr, followed by co-treatment with cuprizone (750 µM) for 42 hr (Table 1). Except for untreated, final dimethyl sulfoxide (DMSO) concentration was 0.1% for all conditions. Dead zebrafish were counted every day and removed. Then, at 4 days post fertilization (dpf) stage, total lethality was calculated. The experiments were performed three times to determine mean and Standard Deviation (SD) for each condition.

Brain degeneration test:

5 dpf zebrafish were treated with 1000 µM cuprizone for 5 hr. to induce brain degeneration. Final test conditions were: 1) 0.4% DMSO; 2) vehicle; 3) cuprizone (1000 µM); 4) pre-treatment of 4 dpf zebrafish with HIDROX® (400 µg/ml) for 24 hr. followed by co-treatment with cuprizone (1000 µM) for 5 hr., and 5) pre-treatment of 4 dpf zebrafish with hydroxytyrosol (80 µg/ml) for 24 hr, followed by co-treatment with cuprizone (1000 µM) for 5 hr (Table 2). Final DMSO concentration was 0.4% for each condition. At 4 and 5 hr post cuprizone treatment (hpt), brain degeneration, visible as opaque dark brain tissue, was visually assessed and % zebrafish exhibiting brain degeneration was calculated for each condition. Student's t-test was used to analyze data to determine if pre-treatment with 400 µg/ml HIDROX® or 80 µg/ml hydroxytyrosol significantly ($P < 0.05$) decreased % brain degeneration compared to zebrafish treated with cuprizone alone.

RESULTS AND DISCUSSION

Lethality test:

Due to the high acidity of HIDROX® (pH value in aqueous solution is 3.4 - which is known to be toxic to zebrafish) we dissolved the compound into DMSO to generate 100 mg/ml MS. We then conducted lethality test using 5 conditions: 1) untreated; 2) vehicle; 3) HIDROX® (100 µg/ml);4) cuprizone (750 µM) for 42 hr., and 5) pre-treatment with HIDROX® for 6 hr., followed by co-treatment with cuprizone for 42 hr. Untreated and vehicle-treated zebrafish were used as controls. Results are shown in Table 3. 0% lethality was observed for: 1) untreated; 2) vehicle; and 3) HIDROX®

Table 4. Brain degeneration test results (N =30)

	% brain degeneration	
Condition	**4 hpt**	**5 hpt**
1) 0.4% DMSO	0 (0/30)	0 (0/30)
2) Vehicle	0 (0/30)	0 (0/30)
3) Cuprizone (1000 µM)	83 (25/30)	90 (27/30)
4) HIDROX (400 µg/ml) + cuprizone	37 (11/30)	47 (14/30)
5) Hydroxytyrosol (80 µg/ml) + cuprizone	100 (30/30)	100 (30/30)

Numbers in parentheses: number of zebrafish with brain degeneration divided by number of animals per well.

(100 µg/ml), 4) and 5) pre-treatment with HIDROX® for 6 cuprizone for 42 hr. For conditions 4) and 5), Student's t-test showed a significant difference (P = 0.0420), demonstrating that HIDROX® significantly decreased cuprizone-induced lethality in zebrafish. The study also showed that master stock (MS) concentration must be 100 mg/ml in order to achieve adequate dilution factor to reduce the acidic effect of HIDROX®

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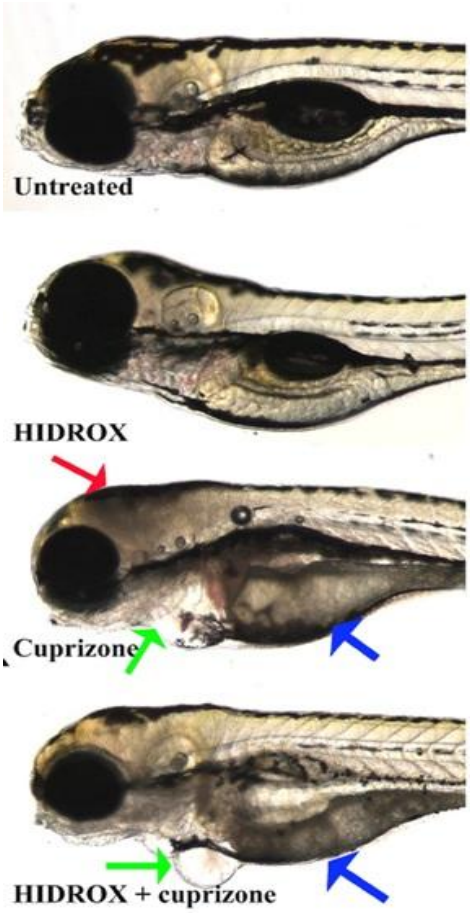
Brain degeneration test:

Using fish water, we diluted master stock (MS) 100 mg/ml, prepared from powder form HIDROX® to generate 400 µg/ml HIDROX®test solution. Effect of 80 µg/ml hydroxytyrosol was also assessed. 4 dpf zebrafish were pre-treated with test compounds for 24 hr, followed by co-treatment with 1000 µM cuprizone for 5 hr as the brain degeneration inducer. 5 conditions were tested: 1) 0.4% DMSO, assay control; 2) vehicle, vehicle control; 3) cuprizone (1000 µM) at 5 dpf stage for 5 hr, brain degeneration inducer; 4) pre-treatment with HIDROX® (400 µg/ml) for 24 hr, followed by co-treatment with cuprizone (1000 µM) at 5 dpf stage for 5 hr., and 5) pre-treatment with hydroxytyrosol (80 µg/ml) for 24 hr, followed by co-treatment with cuprizone (1000 µM) at 5 dpf stage for 5 hr. Brain degeneration (opaque dark brain tissue) was visually assessed at 4 and 5 hr post cuprizone treatment (hpt) using a dissecting microscope. Results are shown in Table 4. 0% zebrafish with brain degeneration was observed for (conditions 1 and 2), and 83-90% brain degeneration was observed for 3) cuprizone (1000 µM) treated zebrafish, validating the assay. 37-47% brain degeneration was observed for 4) pre-treatment with HIDROX® (400 µg/ml) for 24 hr, followed by co-treatment with cuprizone (1000 µM), and 100% brain degeneration was observed for 5) pre-treatment with hydroxytyrosol (80 µg/ml) for 24 hr, followed by co-treatment with cuprizone (1000 µM). For conditions 3) and 4), Student's t-test showed significant difference (P = 0.0005, and 0.0006 for 4 hpt and 5 hpt, respectively), demonstrating that HIDROX® significantly decreased cuprizone-induced brain degeneration in zebrafish. (Figure.1)



Cuprizone treated zebrafish exhibited dark brain (red arrow), pericardial edema (green arrow), and dark intestine (blue arrow). HIDROX + cuprizone did not show dark brain, however, pericardial edema and dark intestine were still present.

Figure1

CONCLUSIONS

HIDROX®,prepared from high concentration (100 mg/ml) MS solution protects zebrafish from cuprizone-induced

lethality and brain degeneration. 80 µg/ml hydroxytyrosol (a dosage of HT ~ 4 times greater than that of the hydroxytyrosol contained in the 400 µg of Hidrox 12% employed for the test) did not protect zebrafish from cuprizone-induced brain degeneration.Because the models we adopted are accepted models in literature to validate the role of compounds in providing neuroprotection. we believe that the study confirms a supportive position for commercially available formulations of olive polyphenol in the management of neurodegenerative-related conditions. Furthermore, the study's conclusion is congruent with current literature findings of the medical proprieties of food bioactive such as olive polyphenols and hydroxytyrosol which warrants further investigation. Suggestive of a functional diversity between natural and purified compounds, the study also highlights the current well-understood relevance of preserving the natural matrix of food bioactive in formulations directed at providing dietary enrichment in human health and wellness. Results also showed that pure hydroxytyrosol did not decrease cuprizone-induced brain degeneration in zebrafish.

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