

## ANNEX

### **Pharmacological and biological properties of the measured compounds**

A summary of the available scientific data for the measured compounds is following:

In total, oleocanthal, oleacein, oleuropein aglycon, ligstroside aglycon present important biological activity and they have been related with anti-inflammatory, antioxidant, cardioprotective and neuroprotective activity.

### **Biological properties of oleocanthal**

Oleocanthal is the dialdehydic form of the ligstroside aglycon without the carboxymethyl group (Mateos R. et al., 2004) which has been related with the “bitter and burning” sensation on the throat, mainly on the base of the tongue that occurs when tasting extra-virgin olive oil.

The pharmacological actions of oleocanthal are the following:

- Action against Alzheimer’s disease

According to scientific research, it has been proved that the soluble oligomers of amyloid-b1-42 peptide act as the basic neurotoxin in the Alzheimer’s disease. As a result, compounds that can modify the formation of these oligomers, referred to as ADDLs, are possible to have therapeutic effects on the AD.

Oleocanthal is of particular interest because of its ability to perturb this particular oligomerisation and reduce the pathogenesis of AD by protecting, at the same time, the neurones from ADDLs’ effects on the synapse pathology. (Pitt J. et. al., 2009). Oleocanthal is also able to inhibit the tau-fibrils formation by modifying covalently the PHF-6 peptide which is of vital importance to their formation. This leads to neurofibrillary tangles which are inherently linked to neurodegenerative diseases as the AD.

The two aldehyde groups of oleocanthal react with the structural unit of lysine to form a complex through the Schiff base formation reaction on the PHF6 peptide, affecting significantly the accumulation of T (tau) proteins in neurofibrils. (Li W. et al., 2009).

It has also been found that oleocanthal interacts with the tau-441 protein causing the stabilization of its secondary structure conformation, thus no further conformation can take place. (Monti M.C et al., 2012)

By examining the oleocanthal action on cell culture, but also on guinea pigs’ brain, it has been discovered that oleocanthal is possible to successfully remove amyloid- $\beta$  ( $A\beta$ ) from the brain, through an increase in the expression and the activity of P-glycoproteins (P-gp) and LDL lipoproteins receptors. LDL lipoproteins bind with proteins-1 (LRP1), which are big  $A\beta$  transfer proteins to the blood-brain barrier.

So, it has been discovered that oleocanthal increases the P-gp and LRP1 activity and consequently the  $A\beta$  removal increases.

As a result, the following conclusion is extracted: potential decrease in the risk of developing AD, which is linked to the extra-virgin oil consumption, could be due to the A $\beta$  removal reinforcement from the brain. (Abuznait A.H., 2013)

- Action against osteoarthritis

Osteoarthritis is a disease whose progression is characterized by increased nitrogen monoxide (NO) production which involves degradation of articulate cartilage.

Recently, oleocanthal isolated from extra-virgin olive oil was found to present anti-inflammatory action similar to that of ibuprofen, a non-steroid anti-inflammatory drug used widely in the therapeutic treatment of common inflammatory diseases.

Oleocanthal and its derivatives reduce the lipopolysaccharides-induced synthesis of NO in cartilage cells without significant effect on the cell viability.

Moreover, oleocanthal intervenes in prostaglandin synthesis through the inhibiting action it exerts on the cyclooxygenase enzymes. (Cicerale S. et al., 2012)

Therefore, oleocanthal can potentially be used as a therapeutic weapon in the treatment of inflammatory degenerative diseases, both in the cure of rheumatoid arthritis and in that of osteoarthritis. (Iacono et al., 2010)

- Inhibition of cyclooxygenase enzymes COX-1 και COX-2

Oleocanthal inhibits cyclooxygenase enzymes in the biosynthetic path of prostaglandins; the latter being inflammatory factors. (Iacono A., 2010)

Oleocanthal has been proved to inhibit cyclooxygenase enzymes in a dose-related manner, imitating the anti-inflammatory action of ibuprofen. (Beauchamp G.K. et al., 2005)

The cyclooxygenase enzymes 1 and 2 (COX 1 and COX 2) are responsible for the transformation of arachidonic acid into prostaglandins and thromboxanes, which are produced as a response to inflammatory or toxic stimuli.

Cyclooxygenase 1 and 2 can be harmful to the human body.

Especially, research made both on humans and on animals, has showed that COX 2 is involved in the pathogenesis of different types of cancer (Harris R.E. et al., 2003, Boland G. et al., 2004, Subbaramaiah K. et al., 2002) and may also play a role in atherosclerosis. (Chenevard R. et al., 2003).

Findings presented by Beauchamp G.K. et al., prove that oleocanthal not only imitates the mechanism of action of ibuprofen, but also presents higher activity compared to the latter, in inhibiting the action of COX 1 and COX 2 in equimolar concentrations.

For example, a concentration of 25  $\mu$ M of oleocanthal inhibited the activity of COX up to 41-57%, whereas, a concentration of 25  $\mu$ M of ibuprofen inhibited only 13-18% of the COX activity.

Furthermore, it has been suggested that long-term intake of small quantities of oleocanthal from extra-virgin olive oil consumption, are in part responsible for the low incidence of diseases associated with a Mediterranean diet.

It is known that low long-term doses of ibuprofen and of other COX inhibitors, like aspirin, have significant benefits on human health as far as the prevention from developing cancer (e.g. rectum and breast cancer) (Garcia-Rodriguez L. A. & Huerta-Alvarez C., 2001, Harris R. E. et al., 2006) and cardiovascular disease (Hennekens C.H., 2002) is concerned.

As a result, long-term consumption of extra-virgin olive oil rich in oleocanthal may contribute to reduction in the incidence of the above diseases. (Cicerale S. et al., 2012)

- **Anti-tumor action / Action against cancer**

Oleocanthal is able to inhibit the proliferation and metastasis of tumor cell lines of human mammary and prostate epithelial cells, and has shown anti-angiogenic activity.

It blocks the phosphorylation of c-Met kinase in vitro with  $IC_{50}$  4,8  $\mu$ M, and may, therefore, have a therapeutic use in the control of malignancies.

It also inhibits proliferation, metastasis and invasion of MCF7, MDA-MB231 and PC-3 tumor cell lines of the breast and the prostate, respectively.

- **Controls skin ageing**

It prevents excessive proliferation of keratinocytes in stress situations due to environmental reasons, by stimulating the synthesis of skin lipids, and genes that are directly related to the differentiation among cells. (Estanove C. et al., 2009).

- **Antimicrobial activity**

Oleocanthal, as proven, cannot be hydrolyzed in the stomach and thanks to this helps inhibit the bacterium *Helicobacter pylori* growth, which is responsible for developing peptic ulcer and certain types of cancer. (Romero C. et al., 2007)

### **Biological properties of oleacin**

Oleacin is a substance with pharmacological actions similar to those of oleocanthal.

- **Anti-oxidant activity**

Oleacin, unlike oleocanthal, has a significant action in that it protects human cells from oxidative stress to a greater extent, even when compared to hydroxytyrosol. (Pavia-Martins F. P. et al., 2009).

- **Angiotensin Converting Enzyme (ACE) inhibitor**

ACE inhibition induces vasodilation; thus, oleacin could potentially be used in the treatment of hypertension and heart failure. (Hansen K. et al., 1996, Somova L.I. et al., 2003)

- Anti-ageing action

Oleacin is a strong anti-oxidant and so it can prevent LDL oxidation, oxidative stress and oxidative damage on DNA.

Hematopoietic endothelial progenitor cells (EPC) are the basic mechanism through which damaged blood vessels are repaired.

These cells are also particularly sensitive to oxidative stress.

Oleacin reduces cell ageing induced by angiotensin II and inhibits the formation of reactive oxygen species (ROS).

This results in a decrease in nitrotyrosine and in oxidative damage on DNA.

Oleacin augments the angiogenic potential of the hematopoietic endothelial progenitor cells *in vitro*.

Oleacin can, therefore, protect the EPCs from oxidative stress caused by angiotensin II (Parzonko A. et al., 2012)

- Heart-protective activity

Oleacin inhibits myeloperoxidase release from neutrophils, which can explain the important role of olive oil in the prevention of cardiovascular diseases. (Czerwińska M. et al., 2012-a)

- Anti-inflammatory action

Oleacin is strong lipooxygenase (LOX) enzyme inhibitor taking part in the inflammation procedure.

### **Biological properties of the monaldehydic form of the oleuropein aglycon**

Chemically, it is classified under ortho-diphenols category, which are a bioactive part in the mechanism of protection of the olive tree fruit from insect attacks and pathogenic bacteria. (Bianco A.D. et al., 1999).

It is also known for its antioxidant action which is comparable to oleuropein.

Moreover, there is evidence that it can prevent further cell proliferation in breast cancer. (Pérez-Trujillo M. et al., 2010).

- Protection from diabetes

Amylin (hIAPP) was discovered in 1987, when isolated from amyloid deposits, taken post mortem from pancreas of patients with type II diabetes.

It is a peptide hormone consisting of 37 amino acids, which is located along with insulin in the secretory granules of the pancreas beta-cells and is secreted in response to trophic stimuli and other secretagogues.

Tests on humans have demonstrated that concentrations of insulin and amylin in plasma raised and lowered parallel, following meals.

The pancreatic amylin deposition is a hallmark of diabetes mellitus type II and there is ample evidence that amylin oligomers exhibit cytotoxic action to the pancreas beta-cells.

The oleuropein aglycon prevents increasing the concentration of amylin in the human body.

It has also been shown that amylin aggregates, grown in the presence of oleuropein aglycon, cannot interact with the cell membrane and destabilize it.

As a result, there is no cytotoxicity in beta-cells. (Rigaccia S. et al., 2010)

- Protection from the Alzheimer's disease

The oleuropein aglycon has been found to be one of the substances responsible for being involved in the protection of the central nervous system from degeneration caused by Alzheimer's disease, by reducing the levels of beta-amyloid, as it has been demonstrated in guinea pigs. (PLOSone 2013, DOI: 10.1371/journal.pone.0071702).