

LC₅₀ Determination and Phenotypic Analysis of Three C₆-C₁ and C₆-C₃ Bio-phenols Using Zebrafish (*Danio rerio*) Model

Diego R. Merchan-Arenas^{*a,b}, Carlos E. Puerto-Galvis^{a,b}, Vladimir Kouznetsov^{‡b}, Carol A. Vargas^{a,b}

^aLaboratorio de Química Orgánica Aplicada, Universidad Manuela Beltrán, Cl. 33 # 26-34, Bucaramanga, Colombia, PBox, 680002. Fax/Tel: +57 7 6525202

^bLaboratorio de Química Orgánica y Biomolecular, Universidad Industrial de Santander, Parque Tecnológico Guatiguara, km 2 vía refugio, Piedecuesta, A.A. 681011, Colombia. Tel: +57 76 344000-Ext. 1243
diego.merchan@docentes.umb.edu.co

Due to the great natural products importance as our more highlighted toolbox to fight against several pathologies, we decide to evaluate the toxicity and reproductive effect of three popular phenols, carvacrol, thymol and eugenol; commonly useful them in pharmacology, cosmetic and food industry.

Zebrafish (*Danio rerio*) model was used to evaluated LC₅₀ and phenotypic changes in embryo stage. The LC₅₀ was calculated for eugenol, 18.7 mg/L; thymol, 7.7 mg/L and carvacrol 5.0 mg/L. Obtained LC₅₀ values were according with early reports for eugenol, 13.2 mg/L. Afterwards phenotypic analysis was carried out to different concentrations and registered to 48, 72 and 96 hpf (hours post fecundation).

For the 48 hpf analysis, eugenol showed a normal growth until 2.337 mg/L where as we could observe, a soft tail curvature, body depigmentation and non-normal yolk sac growth. To the 72 hpf embryos exposed to eugenol showed normal growth to the first concentrations, but its died to 2.337 mg/L and 1.168 mg/L. In the carvacrol molecule, analysis image showed cardiac edema and adverse effects in jaw growth. Embryos exposed to thymol does not showed any reproductive effects.

1. Introduction

Secondary metabolites are called to defend plants from physical, chemical and biological attacks, on this way these compounds also result a great opportunity for humans using them as medical bullets. Plants have given to us a fascinating toolbox of molecules to fight against many diseases, finding structures as alkaloids, coumarins, flavonols, lignans, terpenoids, polyketides, to name a few. Therefore these natural architectures are recognized as bioresources, even as models to get new chemical active entities through of organic synthesis (Newman and Cragg, 2016). One compound of these big family has receiving the attention from 1950's when the free radical theory was proposed, biophenols (Harman, 1957).

Biophenols term was recently coined to refer to these molecules presents in the olive phytochemistry (Romeo and Uccella, 1996) and looking for differentiate it from polyphenols, common polymeric phenols compounds recognized as tannins (Chung et al., 2010). Definition of biophenols establishes that these kind of compounds are mainly biosynthesized in plants through of shikimic acid pathway, reducing the possibility that any nitrogen heteroatom will be include into the final basic structure (Quideau et al., 2011). Several goodness have been attribute to biophenols thanks to its biological properties, observed them in the benefit to uptake a Mediterranean diet, cereals, grains, seeds, olive oil, among others aliments with high phenols content (Visioli et al., 2002).

Biological activities of biophenols have been highlighted in several reports where we can observe it capacity to act as antioxidants (Drózdź et al., 2017), anti-Alzheimer (Omar et al., 2017), anti-proliferative activity (Goldsmith et al., 2015), leishmanicidal (Kyriazis et al., 2013), among others. According to the biophenols phytochemistry, some families of compounds like tannins, stilbenes, phenolic acids, flavonoids, lignans and phenylpropanoids, have been identified. Despite of alkaloids have been seized of the scientific attention in XX century, biophenols have been gained more attention in nowadays (Obied, 2013). Therefore several phenols

have been widely explored recently highlighting that ones are presents in medicinal and aromatic plants. Compounds like eugenol (EU), an exclusive phenylpropanoid (C₃-C₆) obtained from *Eugenia caryophyllus* in at least 12 % of extraction yield. It molecule has showed activities as antifungal (Gill and Holley, 2004), antitumor (Pisano et al., 2007) and antioxidant (Merchán et al., 2011). On the other hand C₁-C₆ phenols as carvacrol (CA) and thymol (THY) have been isolated from oregano yielding 2.7 % of oil with 75 and 6 % of each component, respectively; through of steam distillation process (Missopolinou et al., 2011). These compounds have showed bioactivities as antioxidants (Mastelić et al., 2008), antitumor (Kang et al., 2016) and among others (Suntres et al., 2015).

Despite of it importance like natural biological active compounds and it usage in several food, cosmetics and personal care products, it reproductive risk has not been evaluated. In our research we wanted to evaluate the LC₅₀ of each one of these phenols and its reproductive effects on zebrafish (*Danio rerio*).

2. Materials and methods

All reagents were commercially obtained (synthesis degree) and were used without previous purification. Dry clove bud were acquired in the local market. Gas Chromatograph interfaced to an Agilent Technologies MSD 5963 Selective Detector (MSD) was used for MS identification at 70 eV using a 60 m capillary column coated with HP-5 [5 %-phenyl-poly(dimethyl-siloxane)].

2.1 Toxicity testing and phenotypic screening of biophenols and clove dry bud essential oil, using the zebrafish embryo model

Wild-type adult zebrafish of both sexes were separated in two tanks (30 L each), according to their gender, at 26 ± 2 °C under natural light-dark photoperiods. The fishes were feed twice daily and the water quality was recorded weekly, in order to acclimate the fishes for at least two weeks before experiments begin. For the reproduction of the adult fishes, small breeding tanks were set up in the evening previous to experiment, each containing three males and one female specimen. The tanks were isolated until next morning when the lights switch on and the natural mating occurs, without any perturbation.

The adult fishes were returned to their corresponding tank and the embryos were collected, pooled and washed with E3 medium and transferred into a 92 mm glass Petri dish. Further, dead, delayed, malformed and unfertilized embryos were identified under a dissecting microscope and removed by select aspiration with a pipette. This last procedure was repeated at 12 and 20 hpf in order to remove the unfit embryos. Throughout this period of time, the embryos were kept at 28 ± 2 °C in an incubator under natural light-dark photoperiods.

The selected embryos of 24 hpf from the Petri dish were gently distributed into 96-well plates, placing a single embryo and 200 µL of E3 medium per well.

Adult zebrafish were care and used according to the Guide of the National Institute of Health for Care and Use of Laboratory Animals, keep them healthy and free of any signs of disease. The Ethics and Research Committee of the Heart Institute of Bucaramanga approved the protocol under the Acta Number 050 of May 26 of 2012.

2.2 Determination of zebrafish embryo LC₅₀

For this experiment, in total 72 embryos were required per sample in order to run three independent experiments in three different plates, and each compound was evaluated three times in the same plate, allowing the evaluation of four samples peer plate. In the range of concentrations established by a geometric series, starting from 83.3 and finishing in 1.3 mgL⁻¹, the determination of the LC₅₀ (mgL⁻¹) was based on the cumulative mortality after 72 h of chemical exposure (96 hpf). Each embryo was examined under a dissecting microscope and the statistical analysis was made using Regression Probit analysis with SPSS for windows version 19.0. Data are expressed as the standard error of the mean (SEM) of three different experiments in triplicate.

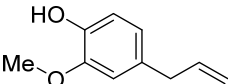
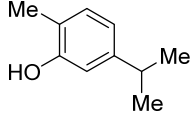
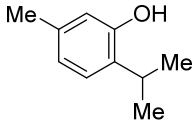
2.3 Phenotypic screening using the zebrafish embryo model

Biophenols were diluted into the E3 screening medium with 1 % V/V of DMSO and aliquots of 200 µL were prepared at concentrations depending on the LC₅₀ of each sample. The surrounding medium (200 µL) was carefully removed from the embryonic plates using an 8-multichannel pipette and then the appropriate chemical aliquot of each compound (200 µL), previously prepared, were added into the corresponding well of the embryonic plate. Eight controls wells were used peer plate, each containing E3 medium with 1 % V/V of DMSO. The embryonic plates were incubated at 28 °C and examined at 48, 72 and 96 hours post-fertilization (hpf) using an OPTIKA zoom stereo microscope (trinocular version of model SZM-1).

3. Results and discussion

Firstly we performed the hydrodistillation of dry bud clove to obtain the essential oil yielding 11.7 % with 60.5 % of eugenol. 7 % of acetyl eugenol and 7.5 % of β -caryophyllene (Merchan et al., 2009). To find the lethal concentration of each biophenols, were evaluated seven concentrations, 83.3 mgL⁻¹, 41.6 mgL⁻¹, 20.8 mgL⁻¹, 10.4 mgL⁻¹, 5.2 mgL⁻¹, 2.6 mgL⁻¹ and 1.3 mgL⁻¹. We found the LC₅₀ for commercial compounds, carvacrol, eugenol, thymol and for dry bud clove essential oil (Table 1).

Table 1: Biophenols LC₅₀ values.

Sample	Structure	LC ₅₀ (mgL ⁻¹)
Dry bud clove essential oil	---	13.2
Eugenol		18.7
Carvacrol		5.0
Thymol		7.7

Eugenol and dry bud clove essential oil (DBCEO) toxicities have been evaluated in several aquatic organisms due to its anesthetic activity, widely employed in fishes management (Hajek et al., 2006). In our research the LC₅₀ value to the eugenol was 13.2 mg/L for 96 hpf, this value was according with the LC₅₀ found after 168 hpf, 15.64 mg/L in zebrafish (Máková et al., 2008). DBCEO toxicity has been evaluated in other fish species like rainbow trout (*Oncorhynchus mykiss*), 14.1 mg/L (Velišek et al., 2005a); common carp (*Cyprinus carpio*), 18.10 mg/L (Velišek et al., 2005b) and the wells catfish (*Silurus glanis*), 18.40 mg/L (Velišek et al., 2006). As DBCEO toxicity could be attributed to the eugenol we performed this analysis obtaining a LC₅₀ = 18.7 mg/L in zebrafish. As same as DBCEO, this LC₅₀ value was compared with literature where we found similar values (Muñoz-Acevedo et al., 2014). Despite of eugenol is a natural product widely used in food, cosmetic and biomaterials application, it has been showed hepatotoxic, genotoxic and carcinogenic effects (Maralhas et al., 2006). Genotoxic molecular bases were described in early studies by Tsai and coworkers in a theoretical study related with the genotoxic activity and structural features of several alkylbenzenes. Allylic fragment result to be an important moiety where occurs hydroxylation and sulfation activation towards formation of carbonion ion, responsible of the adverse effects in the cell (DNA) (Tsai et al., 1994).

In the carvacrol and thymol case, we didn't find reports about LC₅₀ evaluation in fishes, however, some studies showed toxic activity in larvae (c. *Pipiens molestus*) reaching values as 37.6 μ gL⁻¹ and 36.0 μ gL⁻¹, respectively. In a recent study Radwan and coworkers found LC₅₀ values using mosquito larvae (*Culex pipiens*) for these two phenols where carvacrol showed 44.38 μ gL⁻¹ and thymol 37.95 μ gL⁻¹ of LC₅₀. Structural relationship between carvacrol and thymol showed that hydroxyl position had a relevant effect over toxicity. Insecticidal toxicities against *Pochazia shantungensis*, showed a higher toxic effect when nymph was exposure to the carvacrol (56 mgL⁻¹) attributed to the methyl position (Park et al., 2017). However according to our results thymol showed higher toxicity value than carvacrol. It could be attributed to the action mechanism in the zebrafish is different than mosquito larvae and functional groups have another kind of interaction with macromolecules (Jeon et al., 2009). Despite of action mechanism has not described and understood, some proposals establish that those compounds permeate the mitochondrial membrane rising a pro-oxidative effect inducing cell apoptosis (Deb et al., 2011; Yin et al., 2012).

Taking the LC₅₀ of each compound we performed phenotypic analysis on zebrafish, using five different concentration under LC₅₀ values. We identified damages through of embryo development to 48, 72 and 96 hpf. Some samples showed several effects in features as curved bodies (CB), jaw malformation (JW), delayed hatching (DH), yolk sac edema (YSE), mild intestine (MI) and pericardial edema (PE). Most effects were found to low concentrations, higher concentrations to 96 hpf kill fishes. In the case of EU does not produce any body defect (Table 2).

Table 2 Summary of effects of biophenols on zebrafish embryos after 96 hpf

Comp.(mgL ⁻¹)	Morphological defects ^a				
	Curved bodies	Jaw malformation	Yolk sac edema	Mild intestine	Pericardial edema
DBCEO (0.812)	+	-	++	-	-
DBCEO (1.625)	+	-	-	-	+
DBCEO (3.250)	+	+	-	-	+
CA (0.312)	-	+	-	+	-
CA (0.625)	-	+	-	+	+
CA (1.25)	+	+	+/-	-	+
TH (0.432)	-	+	-	-	+
TH (0.875)	-	+	-	-	+
TH (1.750)	-	+	-	-	++
Blank ^b	-	-	-	-	-

^a +++++ = very severe effect (75–100%); +++ = severe effect (50–75%); ++ = moderate effect (25–50%); + = minimal effect (5–25%); +/- = either minimal or no effect (0–5%); - = no effect ^b E3 medium without DMSO.

Initially we check reproductive development through of phenotypic analysis to 48 hpf. Eugenol showed a normal development until 2.337mgL⁻¹, where it shows a soft body curvature, body and eyes depigmentation and a yolk edema. To a 1.168 mgL⁻¹ equally we could observe same effects on embryo growth. In the case of DBCEO composed mainly by eugenol we could observe almost the same behavior where embryos showed yolk reduction, eyes and body depigmentation. To a 6.5 mgL⁻¹, column deviation towards yolk and movement difficulties. Carvacrol and thymol don't shown phenotypic changes (Figure 1).

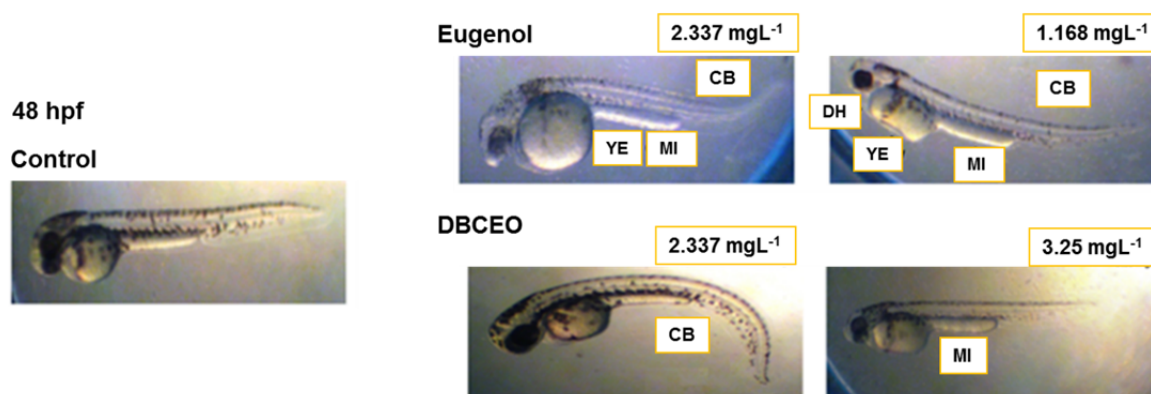


Figure 1: Phenotypic changes induced by eugenol and DBCEO to 48 hpf.

Afterwards we checked embryo development to 72 hpf however, body defects were as 48 hpf, showing body curvature, attribute mainly to the hepatotoxic activities and depigmentation related with inflammatory effects through of metabolic activation mediated by P450 cytochrome in the skin, forming allergenic compounds (Hagvall et al., 2008).

Finally we observe phenotypic changes to 96 hpf where the eugenol doesn't produce any morphological change in the embryo. DBCEO showed same effects where curvature body was the most highlighted result affecting the embryo movement and tail was not formed completely and jaw malformation was also identified. In the case of carvacrol it shows pericardial edema effect and jaw malformation no registered in table 2 because this effect was not normally observed. Thymol exert pericardial edema as well that could be associated with the COX-2 gene expression (Dong et al., 2010). On the other hand, increasing yolk edema and extension could be a protection mechanism where cells recycled fat acids of the membranes improving the lipids deposit (Gubern et al, 2009).

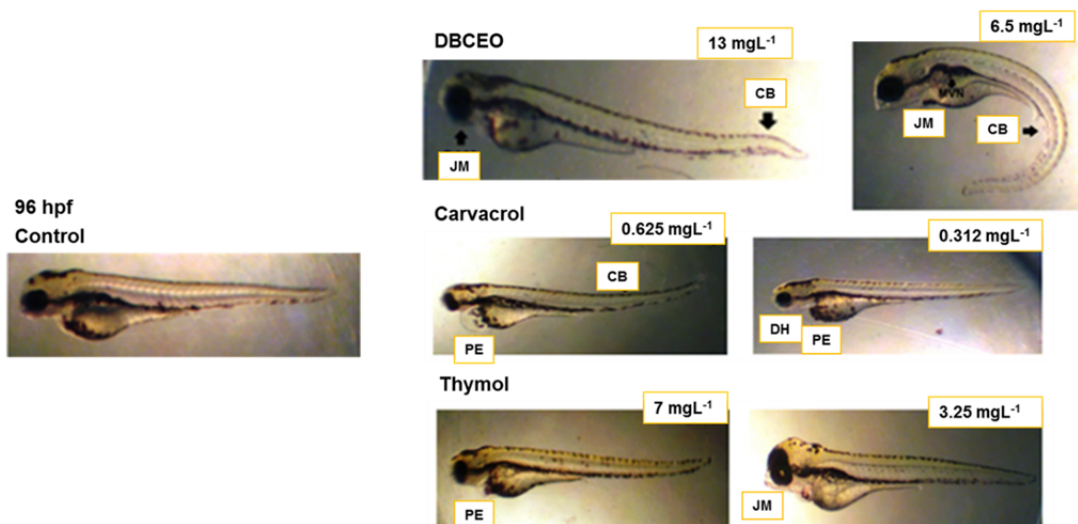


Figure 2: Phenotypic changes induced by DBCEO, carvacrol and thymol to 96 hpf.

4. Conclusion

We evaluated toxicities of three biophenols widely used in food and cosmetic as flavoring, eugenol, carvacrol and thymol. These compounds showed toxicities values from 18.7 -5.0 mgL⁻¹ where carvacrol was the most toxic biophenol and lower effects were observed for eugenol (18.7 mgL⁻¹). Phenotypic analysis afforded to observe that several reproductive effects can be acquired when embryos are exposed to these molecules. Main alteration were observed in the body curvature, especially by DBCEO, jaw malformation exert by thymol and pericardial edema mostly observed for carvacrol.

Acknowledgments

Authors are grateful for the financial support by the UMB, Grant No. CB-2015-01, and for the financial support from Patrimonio Autónomo Fondo Nacional de Financiamiento para la Ciencia, la Tecnología y la Innovación, Francisco José de Caldas, contract RC-0346-2013. DM and CP thanks COLCIENCIAS for the doctoral fellowship.

References

- Chung K. T., Wong T. Y., Wei C. I., Huang Y. W., Lin Y., 1998, Tannins and human health: a review. *Crit. Rev. Food. Sci. Nutr.* 38, 421-464.
- Dong W., Matsumura F., Kullman S. W., 2010, TCDD Induced Pericardial Edema and Relative COX-2 Expression in Medaka (*Oryzias Latipes*) Embryos. *Toxicol. Sci.* 118, 213-223.
- Drózdź P., Sentkowska A., Pyrżyńska K., 2017, Biophenols and antioxidant activity in wild and cultivated heather. *Nat. Prod. Res.* 31, 1181-1184.
- Gill A. O., Holley, R. A., 2004, Mechanisms of Bactericidal Action of Cinnamaldehyde against *Listeria monocytogenes* and of Eugenol against *L. monocytogenes* and *Lactobacillus sakei*. *Appl. Environ. Microbiol.* 70, 5750-5755.
- Goldsmith, Ch. D., Vuong, Q. V., Sadeqzadeh, E., Stathopoulos, C. E., Roach, P. D., Scarlett, C. J., 2015, Phytochemical Properties and Anti-Proliferative Activity of *Olea europaea* L. Leaf Extracts against Pancreatic Cancer Cells. *Molecules*, 20, 12992-13004.
- Jeon J. H., Lee S. G., Lee H. S., 2015, Isolation of insecticidal constituent from *Ruta graveolens* and structure-activity relationship studies against stored-food pests (*Coleoptera*). *J. Food Protect.* 78, 1536-1540.
- Kang S. H., Kim Y. S., Kim E. K., Hwang J. W., Jeong J. H., Dong X., Lee J. W., Moon S. H., Jeon B. T., Park P. J., 2016, Anticancer Effect of Thymol on AGS Human Gastric Carcinoma Cells. *J. Microbiol. Biotechnol.* 26, 28-37.
- Kyriazis J. D., Aligiannis N., Polychronopoulos P., Skaltsounis A., Dotsik E., 2013, Leishmanicidal activity assessment of olive tree extracts. *Phytomedicine*, 20, 275-281.

- Máková S., Doleželová P., Pisteková V., Svobodová Z., Bedaová I., Voslarová E., 2008, Comparison of acute toxicity of 2-phenoxyethanol and clove oil to juvenile and embryonic stages of *Danio rerio*. *Neuroendocrinology Letters*, 29, 680-684.
- Maralhas A., Monteiro A., Martins C., Kranendonk M., Laires A., Rueff J., Rodrigues A., 2006, Genotoxicity and endoreduplication inducing activity of the food flavouring eugenol. *Mutagenesis*. 21: 199-204. *Boletín Latinoamericano y del Caribe Plantas Medicinales y Aromáticas*, 13, 336-343.
- Mastelić J., Jerković I., Blazević I., Poljak-Blazi M., Borović S., Ivancić-Baće I., Smrecki V., Zarković N., Brcić-Kostić K., Vikić-Topić D., Müller N., 2008, Comparative Study on the Antioxidant and Biological Activities of Carvacrol, Thymol, and Eugenol Derivatives. *J. Agric. Food Chem.* 56, 3989-3996.
- Merchán Arenas D. R., Muñoz Acevedo A., Vargas Méndez L. Y., Kouznetsov V. V., 2011, Scavenger Activity Evaluation of the Clove Bud Essential Oil (*Eugenia caryophyllus*) and Eugenol Derivatives Employing ABTS+• Decolorization. *Scientia Pharmaceutica*, 79, 779-791.
- Missopolinou D., Tsiptsias C., Lambrou C., Panayiotou, C., 2012, Selective extraction of oxygenated compounds from oregano with sub-critical water. *J. Sci. Food. Agric.* 92, 814-820.
- Muñoz-Acevedo A., Puerto C., Rodríguez J., Aristizábal-Córdoba S., Kouznetsov V., 2004, Estudio químico-biológico de los aceites esenciales de *Croton malambo* H. Karst y su componente mayoritario, metileugenol.
- Newman D. J., Cragg G. M., 2016, Natural Products as Sources of New Drugs from 1981 to 2014. *J. Nat. Prod.* 79, 629-661.
- Obied H. K., 2013, Biography of biophenols: past, present and future. *Functional Foods in Health and Disease*, 3, 230-241.
- Omar S. H., Scott C. J., Hamlin A. S., Obied H. K., 2017, The protective role of plant biophenols in mechanisms of Alzheimer's disease. *J. Nut. Biochem.* 47, 1-20.
- Park P.-Jh., Jeon Y.-J., Lee, Ch.-H., Chung, N., Lee, H.-S., 2017, Insecticidal toxicities of carvacrol and thymol derived from *Thymus vulgaris* Lin. against *Pochazia shantungensis* Chou & Lu., newly recorded pest. *Sci. Rep.* 7, 409-502.
- Pisano M., Pagnan G., Loi M., Mura M. E., Tilocca M. G., Palmieri G., Fabbri D., 2007, Dettori, M. A.; Delogu, G.; Ponzoni, M. and Rozzo, C. Antiproliferative and pro-apoptotic activity of eugenol-related biphenyls on malignant melanoma cells. *Molecular Cancer*, 6, 1-12.
- Quideau S., Deffieux D., Douat-Casassus C., Pouysegu L., 2011, Plant polyphenols: chemical properties, biological activities, and synthesis. *Ang. Chem. Inter. Ed.* 50, 586-621.
- Romeo G., Uccella N., 1996, Olive biophenols: taste supermolecules in Mediterranean aliment culture. *Tetrahedron-Eppos.* 21, 33-80.
- Suntres Z. E., Coccimiglio J., Alipour M., 2015, The bioactivity and toxicological actions of carvacrol. *Crit Rev Food Sci Nutr.* 55, 304-318.
- Tsai R-Sh., Carrupt P.-A., Testa B., Caldwell J., 1994, Structure-Genotoxicity Relationships of Allylbenzenes and Propenylbenzenes: A Quantum Chemical Study. *Chem. Res. Toxicol.* 7, 73-76.
- Velišek J., Svodova Z., Piackvova V., 2005a, Effects of clove oil anaesthetic on rainbow trout (*Onchorynchus mykiss*). *Acta Veterinaria Brno*, 74, 139-146.
- Velisek J., Svodová Z., Piackvova V., Groch L., Nepejchalova L., 2005b, Effects of clove oil on common carp (*Cyprinus carpio* L.). *Veterinary Medicine Czech.* 50, 269-275.
- Velišek J., Wlasow T., Gomulka P., Svobodová Z., Novotný L., Ziomek E., 2006, Effects of clove oil anaesthesia on european catfish (*Silurus glanis* L.). *Acta veterinaria Brno.* 75, 99-106.
- Visioli F., Poli A., Galli C., 2002, Antioxidant and Other Biological Activities of Phenols from Olives and Olive Oil. *Med. Res. Rev.* 22, 65-75.