The Effect of Some Mineral and Phytogenic Additives, Rich in Polyphenols, on Lipid Peroxidation Process

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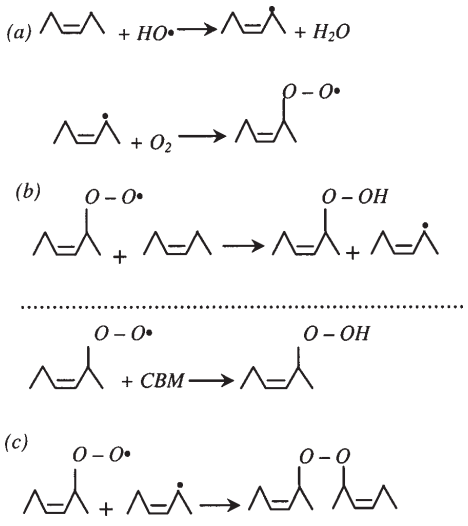
Phenolic compounds show promising and powerful antioxidant properties that can protect both humans and animals against lipid peroxidation. The introduction of plant polyphenols, as plant powders or polyphenolic extracts, in animal diet can solve two major problems: the decrease of oxidative stress in animals and the improvement of nutritive quality of meat and meat products obtained from these animals. In this study it was analyzed the lipid peroxidation process in the liver sampled from weaned pigs, fed with rations in which the addition of Fe, Cu, Mn and Zn derived from inorganic compounds and phytogenic additives (oregano – Origanum vulgare and bilberry fruits - Vaccinium myrtillus). The obtained results show that the highest rate of lipid peroxidation was recorded in case of the samples collected from the animals that received mineral additives in ration (Fe-169,41 ppm, Cu-9,44 ppm, Mn-33,98 ppm, Zn-96,7 ppm) and the introduction of phytogenic additives led to the decrease of lipid peroxidation processes. Due to their antioxidant action, the introduction of oregano and bilberry powders in the ration of pigs determined the decrease of lipid peroxidation process in the liver in the conditions of total or partial replacement of Fe, Cu, Mn and Zn inorganic compounds.

Keywords: phytogenic additives, antioxidant activity, malondialdehyde, oregano, bilberry

Polyunsaturated fatty acids from lipids can be oxidized by enzymatic and non enzymatic pathways. Enzymatic oxidation of lipids consists in direct reactions with molecular oxygen in the presence of different enzymes, from which lipoxygenases and cyclooxygenases are the most studied. Non-enzymatic lipid peroxidation is a radical-chain process involving three sequences: (a) hydrogen atom transfer from unsaturated fatty acid to the chain initiating radical or chain carrying peroxyl radicals, resulting a lipid radical, (b) reaction of the lipid radical with molecular oxygen, resulting a lipid peroxyl radical; reaction of the peroxyl radical with a lipid molecule to give a lipid hydroperoxide and a new lipid radical that continues the radical chain process and (c) reaction of lipid peroxyl radicals with "chain breaking molecule" (CBM), reaction of a lipid peroxyl radical with a lipid radical to give stable peroxides.



The first sequence can be initiated by some reactive oxygen species (ROS), eg. hydroxyl radical, the most reactive radical.

The reactive oxygen species can be molecules like hydrogen peroxide, ions like hypochlorite anion, radicals like hydroxyl radical or superoxide anion, which is an anion and a radical. ROS are generated in living organisms from different enzymatic reactions or in reactions catalyzed by transitional metal ions. Fe(II) and Cu(II) act as catalysts in reactions, facilitating the conversion of hydrogen peroxide into hydroxyl radical, a species frequently proposed to initiate lipid peroxidation:

$$Fe(III) + O_2^{\cdot} \rightarrow Fe(II) + O_2 \quad (1)$$

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + HO^{\cdot} + HO^- \text{ (Fenton reaction)} \quad (2)$$

$$Cu(II) + O_2^{\cdot} \rightarrow Cu(I) + O_2 \quad (3)$$

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + HO^{\cdot} + HO^- \text{ (Fenton-like reaction)} \quad (4)$$

$$H^+ + HO^- \rightarrow H_2O \quad (5)$$

Lipid hydroperoxides (ROOH) are the primary lipid peroxidation products and once formed, in the presence of metal ions, they become susceptible to further free radical chain reaction such as isomeration and decomposition. Their breakage causes secondary products such as pentanal, hexanal, 4-hydroxy-nonenal, malondyaldehyde (MDA). Several studies demonstrated that oxidized lipids in the diet largely determines the levels of circulating lipoproteins and advanced lipid oxidation endproducts in diet are in part cytotoxic and genotoxic compounds [1, 2].

In recent years, phenolic compounds have attracted the interest of researches because they show promising and powerful antioxidant properties that can protect humans

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and animals against lipid peroxidation [3-5]. Plant polyphenols inhibit the enzymes that catalyze peroxidation reactions (lypoxygenase and cyclooxygenase) and show antioxidant activity [6, 7]. The antioxidant properties of plant polyphenols is based on redox properties of hydroxyl groups and the structural relationships between different parts of their chemical structure [8, 9]. Plant polyphenols can act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators [10].

The introduction of plant polyphenols, as plant powders or polyphenolic extracts, in animals' diet can solve two major problems: the decrease of oxidative stress in animals and the improvement of nutritive quality of meat and meat products obtained from these animals.

Industrial breeding of farm animals implies the use in the diet of some mineral additives, generally inorganic compounds, in order to stimulate their development and growth. The latest references data for mineral requirements in pigs were published by USA's National Research Council in 1998. Towards these requirements, in pig's nutrition practice appears an excess of micro-elements, some of them being considered heavy metals (e.g. Cu and Zn). This excess of minerals can be explained by two reasons: the concentration of the minerals in forages is not taken into consideration and, for productive reasons, in the forage there are added mixtures of inorganic compounds (premixes) that lead to the overflow of minerals nutritional requirements.

In this study it was analyzed the lipid peroxidation process in the liver sampled from weaned pigs (10-30 kg), fed with rations in which the addition of Fe, Cu, Mn and Zn derived from inorganic compounds (ferrous sulfate, copper sulfate, manganese oxide and zinc oxide) and phytogenic additives (oregano – *Origanum vulgare* and bilberry fruits - *Vaccinium myrtillus*). The used natural sources (oregano and bilberry) were obtained from plants, respectively fruits harvested from Romania's spontaneous flora (Northern Oltenia, Valcea County). Plant powders were also analyzed in order to determine their concentration in polyphenols and flavonoids and their antioxidant capacity.

Experimental part

The evaluation of phytogenic additives antioxidant capacity

Obtaining vegetal extracts

In order to obtain vegetal extracts, dried plants (oregano and bilberry fruits) were grounded and then subdued to a solid-liquid extraction with ethanol 60%.

Total polyphenols content assay using Folin Ciocalteu reagent

Total polyphenols concentration in the alcoholic extracts was determined using the method described by Singleton [11]. Polyphenols concentration was expressed as mg equivalent gallic acid / 100 mL extract.

Flavonoid content assay

Flavonoid content was estimated by a colorimetric method described in [12]. Flavonoid concentration was expressed as mg equivalent catechin / 100 mL extract.

Reducing power assay

Plant alcoholic extracts' reducing power was determined using the method described in [13]. The results were expressed as mM equivalent $FeSO_4$ /100 mL.

Fe(II) ion chelating capacity assay

The ability of plant alcoholic extracts to chelate Fe^{2+} was determined using the method described in [14]. Absorbances were measured at λ=510 nm and the results, expressed as %Fe(II) chelation, were calculated using the following formula:

$$\%\ Fe(II)\ chelation = \frac{A_c - A_s}{A_c} \times 100 \quad (6)$$

where:

Ac = absorbance of control;

As = absorbance of the sample

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay

DPPH• scavenging activity was determined by the method described in [15]. Absorbances of the samples were measured at λ=517 nm. The scavenging of DPPH radical was expressed as % Inhibition, according to the following formula:

$$\%\ Inhibition = \frac{A_c - A_s}{A_c} \times 100 \quad (7)$$

where:

Ac = absorbance of control;

As = absorbance of the sample

Superoxide anion (O_2•-) scavenging activity assay

The evaluation of superoxide anion scavenging activity of the extracts was based on the method described in [16], with slight modifications. The initiation of the reaction was made by the addition of phenazine methosulfate (PMS); the capacity of the extracts to annihilate superoxide anion was expressed as % Inhibition.

Hydroxyl radical (OH•) scavenging activity assay

The ability of the extracts to annihilate hydroxyl radical was evaluated according to the method described in [17]. The obtained results were expressed as % Inhibition.

Nitric oxide (NO•) scavenging activity assay

The ability of the extracts to annihilate NO· was determined by the method described in [18]. The obtained results were expressed as % Inhibition.

Hydrogen peroxide scavenging activity assay (H_2O_2)

The ability of the extracts to annihilate H_2O_2 was determined by the method described in [19]. The obtained results were expressed as % Inhibition.

The determination of microelements (Fe, Cu, Mn, Zn) in ration

The disintegration of the samples was made using microwaves [20]; the determination of the studied microelements was performed by atomic absorption spectroscopy [21], using a spectrophotometer Thermo Electron+SOLAAR M6 Dual Zeeman Confort (Cambridge, UK).

Animal experiments

The studies were performed on 12 pigs from the Great White breed, with an initial average weight of 12 kg. At the start of the experiments, 3 pigs were anesthetized with diethyl-eter, sacrificed and then there were collected liver samples. From the remaining 9 pigs there were constituted 3 lots, each containing 3 pigs, which were feed for 24 days with rations containing microelements according to the data presented in table 1.

Table 1
RATIONS ADMINISTERED TO EXPERIMENTAL ANIMALS AND THEIR CONCENTRATION IN MICROELEMENTS

Lot no.	Inclusion ratio				Microelement (ppm in ration)			
	Mineral premix*	Zinc oxide	Oregano powder	Bilberry powder	Cu	Fe	Mn	Zn
Lot 1	*1%*	*0*	*0*	*0*	34.44	279.41	64.98	146.70
Lot 2	*0*	*0.5%*	*3%*	*0*	9.70	235.75	35.94	97.68
Lot 3	*0*	*0.5%*	*3%*	*1%*	9.70	235.32	37.80	97.83

*Mineral premix - ferrous sulfate, copper sulfate, manganese oxide and zinc oxide

The first lot received a ration that included microelements (Fe, Cu, Mn and Zn) as inorganic compounds; thus, the levels of these minerals were higher than the ones recommended by USA's National Research Council. Lots 2 and 3 received rations without the mineral premix, but containing oregano and bilberry powders and, in addition, zinc oxide, which led to a decrease of these minerals concentrations in feed.

The evaluation of lipid peroxidation process in experimental animals

Lipid peroxidation was evaluated for liver samples. In order to collect the samples, animals were sacrificed after their anesthesia with diethyl-eter. Liver samples were used for the dosage of malondialdehyde by the method described in [22]. The results were expressed as μmols malondialdehyde (MDA)/g liver.

Results and discussions

The evaluation of phytogenic additives antioxidant capacity

The determination of total polyphenols and flavonoids

The obtained results demonstrate that oregano and bilberry extracts contain mainly the same quantities of polyphenols, but the oregano extract contains a quantity of flavonoids almost double (table 2).

The determination of reducing power

The reducing power of the extracts is presented in table 3. The obtained results demonstrate that the oregano extract had the highest reducing power; bilberry extract had a lower, but significant reducing power.

The determination of Fe(II) ion chelating capacity

In table 3 is presented the ability of the studied plants to chelate Fe(II) ion. The obtained values were appreciable for the two extracts, the highest value being recorded for bilberry extract.

The scavenging of free radicals

Towards the tested free radicals, the two extracts acted differently (table 4). This observation certifies the fact that free radicals annihilation processes depend on the polyphenols type, and not on their concentration. The only radical for which it was recorded a correspondence between flavonoid concentration and scavenging activity was synthetic radical DPPH. The annihilation activity of this radical reflects the global antioxidant activity of the extracts and it is congruent with the reducing power determined towards Fe(III) ion. The studied alcoholic extracts scavenged superoxide anion, the highest inhibition being recorded in case of bilberry extract. This anion, which results in the wake of some enzymatic processes, is not

Table 2
CONCENTRATION OF POLYPHENOLS AND FLAVONOIDS IN OREGANO AND BILBERRY ALCOHOLIC EXTRACTS

Alcoholic extract	Total polyphenols (mg equivalent gallic acid/100 mL)	Flavonoids (μg equivalent catechin/100 mL)
Bilberry	570.02	204.26
Oregano	564.10	396.49

Table 3
REDUCING POWER AND Fe(II) ION CHELATING ABILITY OF THE STUDIED PLANT EXTRACTS

Alcoholic extract	Reducing power (mM equivalent $FeSO_4$/100 mL)	% Fe(II) chelation
Bilberry	49.0	50.64
Oregano	80.8	41.99

Table 4
CAPACITY OF ALCOHOLIC EXTRACTS TO SCAVENGE FREE RADICALS

Alcoholic extract	% Inhibition				
	DPPH•	O_2•–	OH•	NO•	H_2O_2
Bilberry	32.18	21.74	48.52	10.73	20.23
Oregano	63.41	12.61	36.71	54.79	30.92

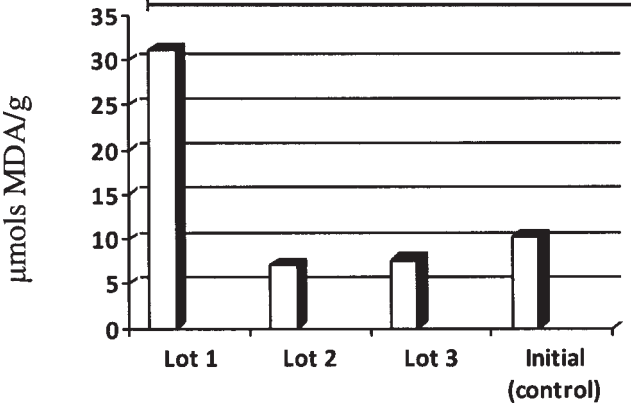

Fig. 1. The concentration of malondialdehyde in liver samples

very aggressive, but, in the presence of water, can generate the most aggressive free radical, hydroxyl radical.

Hydroxyl radical is highly unstable, and, in order to stabilize, can pull out a hydrogen atom from different organic molecules (lipids, proteins, nucleic acids), generating this way chain oxidation reactions upon these molecules. Both extracts scavenged hydroxyl radical, the highest capacity being recorded for bilberry extract.

Nitric oxide is biosynthesized in the cells under the action of nitric-oxide synthase and it has the capacity to react very quickly with superoxid anion to form peroxynitrite ($ONOO^-$), which is an oxidant with properties similar to hydroxyl radical. Towards nitric oxide, oregano extract had a scavenging activity five times higher than bilberry extract.

As for hydrogen peroxide, both alcoholic extracts presented a significant annihilation capacity, mainly oregano extract.

The evaluation of lipid peroxidation process

The obtained results show that the highest rate of lipid peroxidation was recorded in case of the samples collected from the animals that received mineral additives in ration (Fe-169,41 ppm, Cu-9,44 ppm, Mn-33.98 ppm, Zn-96,7 ppm) (Lot 1) (fig. 1). These results are due to the fact that transitional metals ions, in high concentrations, are generators of hydroxyl radicals in Fenton and Fenton-like reactions. Fe and Cu have been recognized as redox active metals in biological systems and they act as pro-oxidants both *in vitro* and *in vivo* [23, 24].

In case of the 2nd and 3rd lots, the exclusion from the ration of inorganic compounds (Fe - ferrous sulfate, Cu - copper sulfate, Mn - manganese oxide), the decrease by 50% of Zn (zinc oxide) and the introduction of phytogenic additives led to the decrease of lipid peroxidation processes. It is noticeable that lipid peroxidation value recorded for the 2nd lot is with 25.29% lower that the one recorded for the initial lot (control lot). These results are due to the cumulative antioxidant effect of zinc and oregano powder. For the 3rd lot, the introduction in ration of bilberry powder had as effect the increase of lipid peroxidation processes by 7.62% comparatively to the 2nd lot. Although in case of the 3rd lot lipid peroxidation process was lower than the one recorded for the initial lot (control), the increase of lipid peroxidation process comparatively to the 2nd lot demonstrates that excessive quantities of polyphenols can have a pro-oxidant effect.

The obtained results demonstrates that phytogenic additives with antioxidant activity significantly decrease lipid peroxidation processes in pig liver in the conditions of total or partial replacement of Fe, Cu, Mn and Zn inorganic compounds. This effect is due to polyphenols capacity to annihilate free radicals and to chelate transitional metals ions. Oregano powder proved to be an additive with strong antioxidant effect, due to its high content in flavonoids, which are the polyphenols with the highest antioxidant activity.

Conclusions

Oregano and bilberry fruits contain polyphenols with antioxidant activity.

The polyphenols from the studied phytogenic additives have the capacity to chelate transitional metals ions and to scavenge free radicals.

Due to their antioxidant action, the introduction of oregano and bilberry powders in the ration of pigs determined the decrease of lipid peroxidation process in the liver in the conditions of total or partial replacement of Fe, Cu, Mn and Zn inorganic compounds.

The use of mineral additives in high concentrations causes lipid peroxidation processes in the liver.

The lowest value of lipid peroxidation was recorded in case of the 2nd lot, which received in ration oregano powder and a supplement of zinc oxide.

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