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STUDY ON THE EFFICIENCY OF CERTAIN PHYTOBIOTICS TO REDUCE THE DETRIMENTAL EFFECTS OF MYCOTOXINS IN POULTRY

PhD thesis

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ABBREVIATIONS

•OH – hydroxyl radical
ADPS – N-Ethyl-N-(3-sulfopropyl)-3-methoxyaniline
AF – aflatoxin
AFB1 – aflatoxin B1
AST/GOT – aspartate aminotransferase/
BW – body weight
CAT – catalase
CD – conjugated diene
ChOD – cholesterol oxidase
CT – conjugated triene
DNA – deoxyribonucleic acid
DON – deoxynivalenol
EFSA – European Food Safety Authority
GGT/γ-GT – gamma-glutamyltransferase
GOD – glucose oxidase
GPO – glycerol-3-phosphate oxidase
GPx – glutathione peroxidase
GR – glutathione reductase
GSH – reduced glutathione
GSSG – oxidized glutathione (glutathione disulfide)
GST – glutathione-S-transferase
H_2O_2 – hydrogen peroxide
HOCl – hypochlorous acid
HPLC – high performance liquid chromatography
L• – lipid radical
LD50 – median lethal dose
LO• – lipid alkoxyl radical
LOO• – lipid peroxyl radical
LOOH – lipid hydroperoxide
MAPK – mitogen activated protein kinase
MDA – malondialdehyde
No. – number
NO• – nitrogen oxide
Nrf2 – nuclear factor erythroid 2–related factor 2
$O_2•^-$ – superoxide anion radical
$ONOO^-$ – peroxynitrite
RBC – red blood cell
RNA – ribonucleic acid
$RO_2•$ – peroxyl radical
ROS – reactive oxygen species
SD – standard deviation
SOD – superoxide dismutase
TNF-α – tumor necrosis factor-α
ZEN – zearalenone

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1. INTRODUCTION

Cereal grains have an important role in the nutrition of monogastric animals and ruminants, and those are frequently infected with toxigenic molds due to field or storage conditions. Their secondary metabolites, the mycotoxins, are potentially toxic substances for animals and humans.

Trichothecene mycotoxins, such as deoxynivalenol (DON) and T-2 toxin, are produced by different Fusarium molds in temperate climatic conditions. They are responsible for most of the mycotoxin problems occurring in Europe and North America, while in subtropical and tropical regions of the world the most important mycotoxins, such as aflatoxin B_1 (AFB_1), are produced by different species of *Aspergillus* genus.

The long-term toxic effects of the above-mentioned mycotoxins on different poultry species are well known, but there is rare information about their short-term effects.

The long-term pro-oxidant effects of T-2 toxin, DON and AFB1 are intensively investigated, resulting sometimes confusing results, but the indicators measured show only the final stage of the process of oxidative stress, which can be related to increasing levels of damage or even adaptation.

The time of the manifestation of the toxic effects of mycotoxins after *per os* intake is an important question, including the level of the oxidative stress induced, and on the other side, the changes in the parameters of the biological antioxidant system.

These are still open questions, therefore one of the aims of the series of experiments presented in my PhD thesis was to evaluate the short-term effects of sub-lethal doses of trichothecene mycotoxins (DON or T-2 toxin) or aflatoxin on the lipid peroxidation processes in broiler chickens or laying hens, and on the amount and activity of the glutathione redox system, an important part of the biological antioxidant defense system.

Phytobiotics represent a wide range of bioactive compounds derived from different parts (leaves, shoot, root, flower and seed) of herbs and spices along with plant extracts or essential oils obtained from the plants, embedded into diets to improve livestock productivity. The importance of these phytobiotics is based on their biologically active components, such as polyphenols, flavonoids etc.

Large scale of bioactive substances has been identified in herbs and spices, with numerous effects. Amongst others, immune stimulating, digestion stimulating, bacteriostatic, antioxidant and microflora balancing activities are the most important ones and already identified in the scientific literature. However, the focus on the use of these is to improve the growth performance of farm animals. Hence, there are many phytobiotic products available as alternatives of antibiotic growth promoters or synthetic antioxidants, and usually these are mixtures of herb essential oils and/or extracts.

There are limited number of studies available about the phytobiotic supplementation on the antioxidant defense-abilities of chickens against the pro-oxidative effects as caused by mycotoxins. In addition, the results are sometimes contradictory due to composition and dosage of bioactive components.

Therefore, the other purpose of my PhD study was to evaluate the effects of a commercially available phytobiotic feed additive (Herbamix™ Basic Premix) - containing essential oils from 7 plants, namely garlic, rosemary, oregano, true cinnamon tree, common thyme, narrow-leaved paperbark and southern blue-gum, and glycerol extract of two plants, namely eastern purple coneflower and Mediterranean milk thistle - on performance traits and some parameters of lipid peroxidation and glutathione redox system of broiler chickens or laying hens.

The bioactive substances of phytobiotics have many potential applications, though the literature often lacks the important and exact information about the details of dosage.

Therefore, the third aim of my work was to investigate the application of the above mentioned Herbamix™ Basic Premix at different concentrations.

2. OBJECTIVES

1. The main objective of my studies was to investigate the effect of mycotoxins (T-2 toxin or DON or aflatoxin) on the initial and terminal phase markers of lipid peroxidation processes, and on the amount and activity of the glutathione redox system in the first 48-hour of mycotoxin exposure in chicken (*Gallus gallus domesticus*), both in broilers and layers.

2. Investigation of the effects of an herbal mixture (Herbamix™ Basic Premix) on the parameters of lipid peroxidation and glutathione redox system of chickens either using it parallelly with trichothecene mycotoxins (T-2 toxin, DON) or aflatoxin exposure, or using it as pre-treatment in different concentrations.

The following experiments were designed, and parameters were measured to achieve the objectives:

I. Effects of herbal mixture and short-term DON or T-2 toxin exposure on performance, lipid peroxidation and glutathione redox parameters in 3-weeks-old broiler chickens:

a) on the lipid peroxidation processes, including the markers of initiation phase, such as conjugated dienes (CD) and conjugated trienes (CT), and on one of the metastable end-products of terminal phase, malondialdehyde, (MDA);

b) on the parameters of the biological antioxidant system, namely glutathione peroxidase (GPx) activity and reduced glutathione (GSH) concentration.

II. Effects of herbal mixture and short-term aflatoxin exposure on performance, lipid peroxidation and glutathione redox parameters of laying hens:

a) on the lipid peroxidation processes, including the markers of initiation phase (CD and CT) and metastable end-product of terminal phase (MDA);

b) on the changes of parameters of the biological antioxidant system (GPx activity and GSH concentration).

III. Effects of herbal mixture pre-treatment and short-term T-2 toxin exposure on performance, lipid peroxidation, glutathione redox and clinical biochemical parameters in broiler chickens:

a) on the lipid peroxidation processes, including the markers of initiation phase (CD and CT) and metastable end-product of terminal phase (MDA);

b) on the changes of parameters of the biological antioxidant system (GPx activity and GSH concentration).

c) on some clinical biochemical parameters of blood plasma, such as concentrations of glucose, cholesterol, triglycerides and uric acid, and activities of aspartate aminotransferase (AST) and gamma-glutamyltransferase (GGT).

IV. Effects of herbal mixture pre-treatment and short-term aflatoxin exposure on performance, lipid peroxidation, glutathione redox and clinical biochemical parameters in broiler chickens:

a) on the lipid peroxidation processes, including the markers of initiation phase (CD and CT) and metastable end-product of terminal phase (MDA);

b) on the parameters of the biological antioxidant system (GPx activity and GSH content).

c) on some clinical biochemical parameters of blood plasma: concentrations of glucose, cholesterol, triglycerides and uric acid, and activities of aspartate aminotransferase (AST) and gamma-glutamyltransferase (GGT).

3. LITERATURE REVIEW

3.1. General view of mycotoxins

Mycotoxins are low-molecular-weight secondary metabolites of filamentous fungi (Bennett, 1987). These metabolites are chemically heterogenous and may cause dose-dependent diverse toxic symptoms in animals and humans. Most of the mycotoxins show overlapping toxicities to invertebrates, plants and microorganisms.

Filamentous fungi or molds produce not only mycotoxins, but antibiotics and phytotoxins too (Bennett and Klich, 2003).

Mycotoxins may have acute or chronic effects, which depends mainly on the type of mycotoxin and on the amount and duration of the exposure. Mycotoxins may cause serious losses in livestock industry by reducing efficacy of animal production resulting significant economic losses (Zain, 2011).

The detrimental effects of the different mycotoxins in animals depend on the species, age, sex, health and nutritional status, and there are potentially synergistic, additive or even antagonistic interactions between them (Kanora and Maes, 2009).

Mycotoxins can contaminate various agricultural products, crops and different feed- and foodstuffs including cereals, nuts, spices, dried fruits, coffee etc. (Miraglia et al., 2008).

Mycotoxin producing fungi genera have variable environmental requirements, some of them favor hot and humid environment, while others may infect the crops at lower temperature. Many factors may also influence their mycotoxin production, but temperature and humidity are the most important determining factors (Filtenborg et al., 1996).

Nowadays, more than 400 different mycotoxins are known, which may have toxic potential, however, only a dozen may have marked toxic effects (Kabak et al., 2006). According to a FAO investigation, approximately 25% of the total feedstuffs in the world are contaminated with one or more mycotoxins (FAO, 2001).

The main route of ingestion of mycotoxin is through the consumption of mycotoxin contaminated plant origin feeds (Bennett and Klich, 2003), but different animal products (e.g. meat, edible offal, milk and eggs) from animals fed with mycotoxin contaminated diet may also have human health concern (CAST, 2003), and inhalation of dust containing mycotoxin is also a possible route of intake (Jarvis, 2002).

There are at least six main groups of mycotoxins, which cause the most common problems worldwide, namely aflatoxins, ochratoxins, trichothecenes, fumonisins, zearalenone and ergot alkaloids.

All of them are produced by different fungi and some species can produce more than one mycotoxin. The most important mycotoxins are produced by three fungal genera, i. e. *Aspergillus*, *Fusarium*, and *Penicillium*.

Mycotoxin producing molds can be divided into two groups, specifically field and storage molds. Field molds, such as *Fusarium* species, produce mycotoxins mainly during their growth on plants in

the cultivation area, whereas storage molds, such as *Aspergillus* and *Penicillium* species, produce mycotoxins after crop harvesting or ensiling the fresh forage, although there are increasing numbers of reports, that due to the climate change they also can infect plants on fields even in continental climate (Dobolyi et al., 2011).

Temperate climatic conditions are favorable for *Fusarium* fungi, which genera may produce fusariotoxins, such as zearalenone (ZEN), fumonisins, and different trichothecenes (deoxynivalenol (DON) and T-2 toxin) (Placinta et al., 1999).

In Europe and North America, the different fusariotoxins are responsible for the vast majority of mycotoxin problems occuring, while in (sub-)tropical regions of the world it is mainly due to the mycotoxins produced by *Aspergillus* and *Penicillium* species (e.g. aflatoxin B_1 (AFB_1) and ochratoxin A (OTA)) (Duarte et al., 2010).

As mentioned above, this situation has been changed during the last decade due to the change of climatic conditions in different areas of the world, some - in the given region previously unknown - molds can produce mycotoxins, such as the OTA producing *Penicillium verrucosum* (Duarte et al., 2010) or the AFB_1 producing *Aspergillus flavus* (Dobolyi et al., 2011).

In a recent paper Monbaliu et al. (2010) reported that 82% of European crop samples are contaminated with detectable amount of mycotoxins.

In overview 50% of crop samples in Europe are contaminated with DON at low doses and 75-100% of the samples showed contamination of more than one mycotoxin (Streit et al., 2012).

In a recent survey, BIOMIN published the risk levels of the different mycotoxins in several parts of the World. Risk level expresses the percentage of the analysed samples, which were positive for at least one mycotoxin, which means their concentration were higher than the threshold level. This threshold level for DON, T-2 toxin and for aflatoxin was 150 µg/kg, 50 µg/kg, and 2 µg/kg, respectively (BIOMIN World Mycotoxin Survey, 2017).

In case of DON in Central Europe 86%, and in Northern Europe 79% of the analysed samples exceeded the threshold level, while in case of T-2 toxin the same indicator was 35% and 49%, respectively. Aflatoxin was found over its threshold level in 78% of the samples from South Asia (eg. India), and in 54% of the samples from South-East Asia. The co-contamination of the different mycotoxins in the samples was also very high (76% worldwide) (BIOMIN World Mycotoxin Survey, 2017).

3.2. Fusarium mycotoxins

With more than 140 known metabolites, the *Fusarium* mycotoxins (fusariotoxins) are the largest group of mycotoxins. They are synthetized by many species belonging to the *Fusarium* genus (Sobrova et al., 2010). These field molds mostly infect the cereals, causing *Fusarium* head blight (FHB) in wheat, and ear rot in maize. The most important species are *F. graminearum*, *F. poae* and *F. culmorum* (Rocha et al., 2005), which are usually produce T-2 toxin or DON. *F. culmorum* and *F. graminearum* can also synthetize zearalenone (ZON) which is also produced by *F. crookwellense*, *F. cerealis*, *F. equisiti* and *F. semitectum* (Zinedine et al., 2007). Fumonisins are produced by *F. verticillioides* (formerly *F. moniliforme*), *F. proliferatum* and *F. nygamai* (Thiel et al., 1991). Beauvericin (BEA) and enniatins (ENNs) are mainly produced by *F. avenaceum*, *F. tricinctum*, *F. poae* and *F. culmorum* (Logrieco et al., 2002).

I have done my research with mycotoxins belonging to the trichothecene fusariotoxins, so in the followings I would like to concentrate on this group.

3.2.1. Trichothecene mycotoxins

Nowadays, more than 60 naturally occurring trichothecenes are described. All of them have a tricyclic nucleus, named trichothecene, and contain an epoxide group between the 12^{th} and 13^{th} carbon atoms, which is responsible for their toxic (pro-oxidant) characteristics (Hussein and Brasel, 2001). Trichothecene mycotoxins have low-molecular weight, around 200-500 Da (Pestka, 2007).

Not only *Fusarium* species can synthetize trichothecene mycotoxins, but also species belonging to *Myrothecium*, *Trichoderma*, *Trichothecium*, *Cephalosporium*, *Verticimonosporium*, and *Stachybotrys* genera.

The chemical differences between these different sesquiterpene metabolites vary in both the position and number of hydroxylation, as well in the position and complexity of esterification (Bamburg, 1976).

The different trichothecenes can be categorised into four groups (Kimura et al., 2007).

In the trichothecene skeleton, there are five positions at which side chains can be added (C-3, C-4, C-15, C-7, and C-8). In case of 'type A' trichothecenes a single bond can be found at C-8, in case of 'type B' trichothecenes there is a carbonyl- (keto-) group at C-8.

'Type A' trichothecenes include T-2 toxin, HT-2 toxin and diacetoxyscirpenol (DAS). The most important 'type B' trichothecenes are DON and its derivatives, 3-acetyl deoxynivalenol (3-acDON), 15-acetyl deoxynivalenol (15-acDON), and nivalenol (NIV) (Puri and Zhong, 2010).

'Type C' trichothecenes contain another epoxy- group between the 7^{th} and 8^{th} or 8^{th} and 9^{th} carbon atom, while 'type D' trichothecenes have a macrocyclic ring between C-4 and C-15 (Wu et al., 2010). Crotocine belongs to 'type C' (Wu et al., 2010), while satratoxin G is a macrocyclic 'type D' trichothecene mycotoxin produced by *Stachybotrys chartarum* (Chung et al., 2003).

The most important structural features of trichothecenes affecting their biological activities are the followings:

1) The double bond between C-9 and C-10 is essential for toxic effects and biological activity of trichothecenes. The reduction of this double bond results in a substantial decrease in toxicity

2) The presence of the 12, 13-epoxide ring as a typical structure for trichothecene toxicity, since the epoxide ring is necessary for inhibition of protein synthesis.

3) The five variable R groups, the structure and position of a side chain, the presence of a second epoxy ring in 'type C' trichothecenes such as crotocine that have another epoxy group at C-7 and C-8 position.

4) 'Type D' trichothecenes, such as satratoxins and roridins include a macrocyclic ring at C-4 and C-15 (Wu et al., 2013; Bräse et al., 2009).

F. sporotrichioides and *F. poae* produce mainly 'type A' trichothecenes, such as T-2 toxin, while *F. culmorum*, *F. graminearum*, *F. crookwellense* and *F. sambucinum* synthetise mainly diacetoxyscirpenol (DAS) and deoxynivalenol (DON) (Lauren et al., 1987).

The biosynthesis of the different trichothecenes proceeds from trichodiene, and involves a sequence of oxygenation, isomerization, cyclization and esterification, requiring 10 steps for deoxynivalenol (DON) formation, 12 steps for diacetoxyscirpenol (DAS) and 14 steps for forming the most complex metabolite, T-2 toxin (Desjardins et al., 1993).

3.2.2. T-2 toxin

3.2.2.1. Chemical structure and metabolism of T-2 toxin

T-2 toxin, and its main bioactive metabolite, HT-2 toxin is 'type A' trichothecene mycotoxins. Their trichothecene skeleton is chemically stable and the 12,13-epoxide ring is resistant to nucleophilic attack.

These mycotoxins are non-volatile, highly resistant to UV light and heat (Sokolovic, 2008) and are not degraded during feed and food processing (Eriksen, 2003).

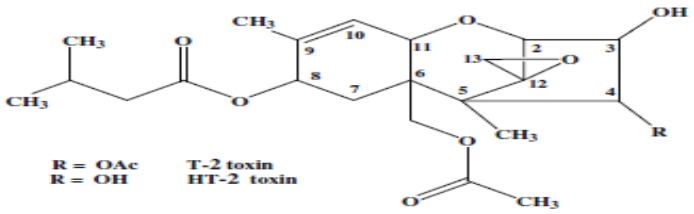
T-2 toxin and HT-2 toxin are also stable at neutral to acidic pH (Ueno, 1987), and consequently, they are not hydrolysed in the stomach after ingestion (Eriksen, 2003).

T-2 and HT-2 toxins are produced by *Fusarium sporotrichioides*, *F. acuminatum, F. equiseti* and *F. poae*. They often occur together in infected cereal crops (wheat, maize, barley, oat, and rye) and in processed grains (Zöllner and Mayer-Helm, 2006).

T-2 toxin is rapidly metabolized to a variety of metabolites (Yoshizawa et al., 1980). Different studies and animal models have shown that the major metabolic pathway of T-2 toxin by hepatic microsomal carboxylesterase is hydrolysis, which occurs at the C-4 position (C-4 deacetylation), resulting HT-2 toxin, the major *in vivo* metabolite (Dohnal et al., 2008).

Structures of both T-2 toxin and HT-2 toxin are illustrated in Figure 1.

Figure 1. Chemical structure of T-2 toxin and HT-2 toxin (Wang et al., 2012)



HT-2 toxin can be hydroxylated to 3'-OH-HT-2 in the liver or can be hydrolyzed to T-2 tetraol via T-2 triol. It is also possible that T-2 toxin is hydroxylated immediately to 3'-OH-T-2 (Bergmann et al., 1988). De-epoxidation and biosynthetic conjugation to glucuronic acid of the different compounds can also be a metabolic pathway (Yoshizawa et al., 1980; Wu et al., 2010).

Several uncharacterized metabolites of T-2 toxin were also found, especially in poultry (Young et al., 2007). Some metabolites are equally (e.g. HT-2 toxin) or even more toxic than T-2 toxin (Dohnal et al., 2008). Increased toxicity of T-2 toxin has even been observed owing to enterohepatic circulation (Coddington et al., 1989; Sokolovic et al., 2008). Retention of T-2 toxin in bile has been observed in broiler chicken, indicating the important role of the biliary excretion in the elimination of this mycotoxin (Chi et al., 1978).

3.2.2.2. Toxicity of T-2 toxin

T-2 toxin has various adverse effects on farm animals. T-2 toxin and its main metabolite, HT-2 toxin are well-known inhibitors of protein, RNA and DNA synthesis (Murugesan et al., 2015). They bind to the 60S subunit of eukaryotic ribosomes and impair the function of peptidyl transferase (Feinberg and McLaughlin, 1989). Depending on the substituent, there are two types ('type-E' and 'type-I') of mechanisms of protein synthesis inhibition (Ehrlich and Daigle, 1987). 'Type-E' is responsible for inhibition of elongation as well as termination of protein synthesis, whereas 'type-I' is responsible for inhibition of initiation of protein synthesis. T-2 toxin and HT-2 toxin are 'type-I' inhibitors, while DON belongs to the 'type-E' inhibitors.

T-2 toxin is also cytotoxic and genotoxic, and has pro-oxidant properties, therefore, it largely affects the performance of animals (Sokolovic et al., 2008).

T-2 toxin is also responsible for inducing apoptosis (Yang et al., 2000) and it regulates cytokine production (Ouyang et al., 1995).

Murugesan et al. (2015) described that it causes reduction in the weight of bursa of Fabricius, causes peroxidative changes in the liver, abnormal blood coagulation and hypoproteinaemia. In poultry species oral lesions, reduced growth, abnormal feathering, decreased egg production and lower egg shell quality are also documented. In geese, Ványi et al. (1994a) reported 50% reduction in the hatching rate at 0.2 mg/kg/day T-2 toxin exposure for 18 days and also decrease in the egg yield. As a result of T-2 toxin administration Ványi et al. (1994b) also reported involution of the oviduct, lymphocyte depletion, necrosis and amyloidosis in the spleen, catarrhal enteritis, signs of colloid stasis

in the thyroid gland and large numbers of secretory granules in the cytoplasm of the adrenaline-producing cells of the adrenal gland in laying geese.

In case of poultry the effects of T-2 toxin / HT-2 toxin are mostly dependent on time, on the applied dose and on the presence of other mycotoxins.

T-2 toxin affects the immune system, which causes decrease in leukocyte counts (leukopenia) or reduced antibody formation after vaccination (Creppy, 2002).

Sokolovic et al. (2008) described that in 7-day-old broiler chickens the LD_{50} of T-2 toxin was 4.97 mg/kg, while Chi et al. (1978) described a LD_{50} value in broiler chickens 6.3 mg/kg BW.

3.2.2.3. Effect of T-2 toxin in chickens

T-2 toxin and DON toxicity is variable in different poultry species. For instance, the order of sensivity for T-2 toxin is goose> duck> chicken (Mézes et al., 1998). The marked differences are related to severity and extent of changes.

T-2 toxin is the most acutely toxic trichothecene and the exposure can occur through different routes (Sokolovic et al., 2008).

In chronic exposure T-2 toxin and HT-2 toxin shows various toxic effects like diarrhea, lesions in oral cavity, gizzard and instestine (Wyatt et al., 1973), also liver damage, weight loss and emesis (Li et al., 2011). Sokolovic et al. (2008) investigated the effects of feeding T-2 toxin in concentrations around 1 to 5 mg/kg for one week, which resulted in lesions in the mouth.

In laying hens common symptoms of chronic T-2 toxin exposure are the impaired feathering, reduced egg production and impaired hatchability (Diaz et al., 1994).

T-2-toxin also has neurotoxic effects and it even destroys the blood-brain barrier resulting increase in indoleamin (e.g. serotonin) concentration of the brain (Wang et al., 1998), which causes feed refusal and abnormal behavior (MacDonald et al., 1988).

3.2.3. DON (deoxynivalenol)

3.2.3.1. Chemical structure and toxicity of DON

DON is one of the most important trichothecene mycotoxin due to its high prevalence in cereals (Rotter et al., 1996), and is commonly produced by several phytopathogenic *Fusarium* species, such as *Fusarium graminearum* and *Fusarium culmorum* (Turner, 2010). Fungi producing 'type B' trichothecenes can be grouped into two chemotypes based on whether they produce DON (or acetylated derivatives, as 3-acDON, and 15-acDON) (Logrieco et al., 2002) or nivalenol (NIV) (Lee et al., 2011).

The occurrence of DON in feeds from Central and Northern Europe represents more than 75% of the total number of samples (BIOMIN World Mycotoxin Survey, 2017) and it is a potential marker of the occurrence of other mycotoxins (Sobrova et al, 2010).

Chemically, DON is a 12,13-epoxy-3α,7α,15-trihydroxytrichothec-9-en-8-on (Nagy et al., 2005). Its molecule contains three free hydroxyl groups, which are associated with its toxicity.

Physico-chemically, DON is also very heat-stable. An investigation was done between 170°C to 350°C, with no reduction of DON concentration after 30 min at 170°C (Sugita-Konishi et al, 2006). However, since DON is water-soluble, it may leach into the cooking water (Visconti et al., 2004).

DON is less toxic than T-2 toxin, however, extremely high DON doses can cause shock-like death.

LD_{50} for 10-day old duckling is 27 mg DON/kg BW when administered subcutaneously (Yoshizawa and Morooka, 1973) and 140 mg/kg feed for day-old chicks (Huff et al., 1981; Pestka, 2007).

The first target of DON toxicity is the intestinal tract, as DON impairs the intestinal barrier functions by affecting the expression and assembly of tight junctions and initiating an inflammatory response (Pinton and Oswald, 2014).

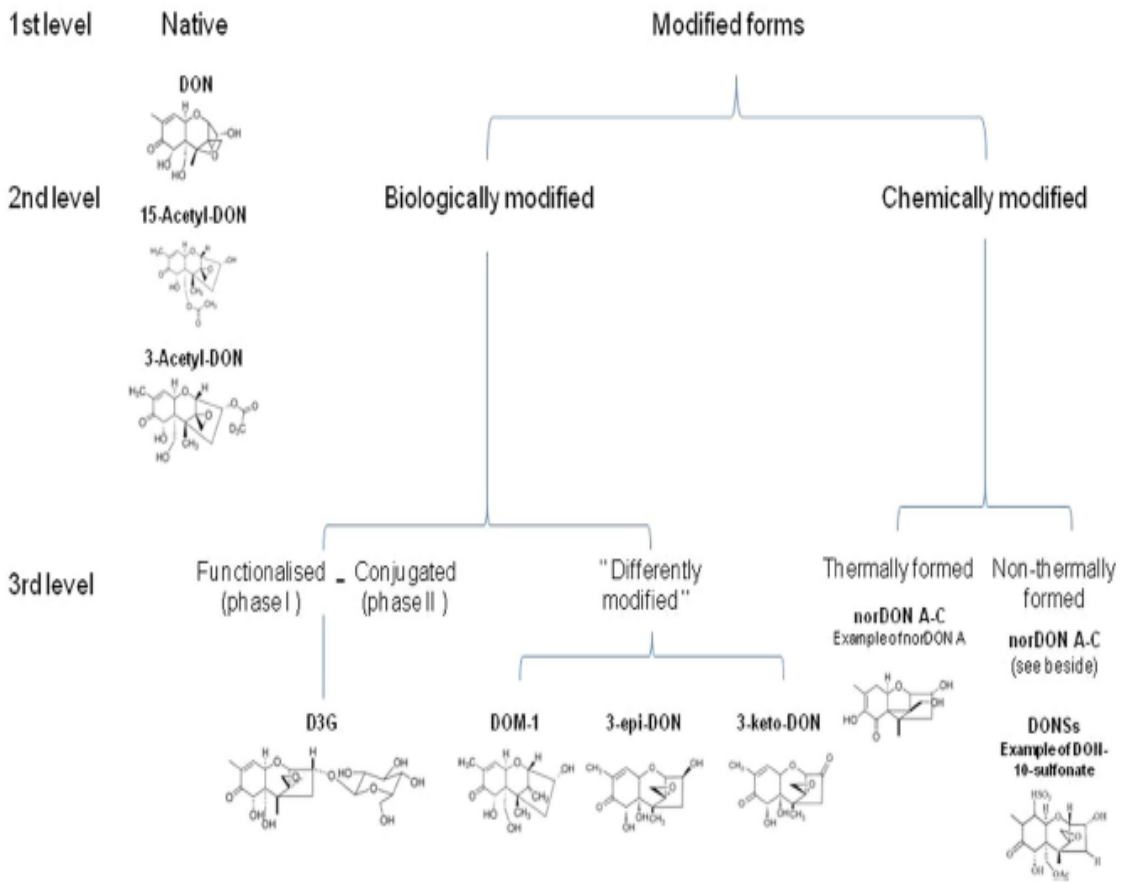
After ingestion, de-epoxidation of DON to deepoxy-deoxynivalenol (DOM-1) occurs mostly in proximal part of the small intestines (Awad et al., 2011). The formed non-toxic DOM-1 is absorbed and subsequently excreted via the kidneys (Heyndrickx et al., 2015).

DON producing *Fusarium* species may also synthesize acetylated derivatives of DON, 3-acetyl-deoxynivalenol (3acDON) and 15-acetyl-deoxynivalenol (15acDON) (De Boevre et al., 2012). Besides these fungal derivatives, animals and humans are also exposed to plant derived DON-glucosides, including DON-3-β-d-glucoside (DON3G). These conjugates are expected to have a low toxicity, but may contribute to overall exposure (Alizadeh et al., 2016).

According to Pestka (2007), both 3-acDON and 15-acDON are thought to be less toxic than DON for a long time. But in a recent study Pinton et al., (2012) reported that 15-acDON is more toxic than DON and 3-acDON both *in vitro* and *in vivo*.

The structures of DON and its metabolites are illustrated in Figure 2.

Figure 2. Chemical structure of DON and its metabolites (Payros et al., 2016)



3.2.3.2. DON mycotoxicosis in chickens

DON (formerly called vomitoxin) causes vomiting and feed refusal in pigs (SCF, 1999). The emetic effect of DON is thought to be mediated through affection of the serotonergic activity in the central nervous system or via peripheral actions on serotonin receptors (SCF 1999). Taste aversion has also been reported (Prelusky and Trenholm, 1993).

However, in broiler chickens, the feed refusal is not so severe as in pigs. The reason for this is that in broilers, higher cerebral levels of norepinephrine was found after ingestion of DON, antagonizing the effect of serotonin on appetite depression (Swamy et al., 2004).

Examining the mechanism of DON-induced diarrhea, it was found to be associated with the inhibition of D-glucose associated water absorption in intestinal cell lines (Maresca et al., 2002). After DON exposure Awad et al. (2008) found decreased glucose uptake from the intestine in chickens.

According to the results of Prelusky et al. (1994) the degree of susceptibility of different species is the following: pigs > mice > rats > poultry ≈ ruminants (Prelusky et al., 1994).

There are differences in pharmacokinetic like absorption, distribution, metabolism and excretion of DON within animal species, which might be responsible to differential sensitivity.

Acute mycotoxicosis caused by DON is rare in poultry. LD_{50} of DON in acute toxicity is set at 140 mg DON/kg BW in broiler chickens (Huff et al., 1981).

While in case of chronic exposure at low or moderate levels, DON can cause altered feed efficiency, anorexia, diarrhea and lower weight gain (Pestka and Smolinski, 2005).

A lot of investigations have been carried out with broiler chickens to investigate the effect of different feed-borne DON concentrations on production traits, such as feed intake and body weight (or weight gain). In some experiments, no feed refusal and no decrease in body weight were observed (Bergsjo and Kaldhusdal, 1994, Kubena et al., 1997), while others registered decrease in both parameters (Dänicke et al., 2007, Yunus et al., 2012). These results are very variable and even contradictory, so it is difficult to make dose-response relationship between growth depression and dietary concentrations of DON. Moreover, there are differences between the effects observed in case of experimental versus natural contamination. In most cases, naturally contaminated feed causes more expressed negative effects (Rotter et al., 1996), which may be due to the presence of more than one mycotoxins or other potentially toxic compounds in the contaminated feed (Smith et al., 1997).

According to Awad et al. (2011) it has to be mentioned that broiler chickens may adapt to DON contaminated feed, possibly due to changes of the intestinal microbiota resulting in more active de-epoxidation before absorption.

3.2.3.3. Molecular mechanism of action of DON and T-2 toxin

Mostly all trichothecenes have a close affinity for the 60S subunit of ribosomes, which causes inhibition of initiation, elongation and termination steps of protein synthesis (Rocha et al., 2005). T-2 toxin acts on initiation phase and DON has effect on elongation and/or termination step (Awad et al., 2008; Sokolovic et al., 2008).

T-2 toxin and DON also affects RNA and DNA synthesis and shows adverse effects on the mitochondrial function (Minervini et al., 2004; Ueno, 1984).

Apoptosis or programmed cell death are also induced by trichothecenes both *in vivo* and *in vitro* (Pestka et al., 2004; Yang et al., 2000; Minervini et al., 2004). According to the findings of Shifrin and Anderson (1999) T-2 toxin is a weak, while DON is a strong inducer of programmed cell death.

Trichothecenes are able to induce production of reactive oxygen species (ROS), causing oxidative stress and thus membrane and DNA damage (Atroshi et al., 1997; Leal et al., 1999; Minervini et al., 2005; Rizzo et al., 1994; Vila et al., 2002).

Considering the results of investigations on the relationship between DON or T-2 toxin and oxidative stress are contradictory.

According to El Golli-Bennour and Bacha (2011) T-2 toxin is a moderate pro-oxidant, while DON is a non-prooxidant mycotoxin.

Rezar et al. (2007) observed DNA fragmentation of leukocytes in broiler chickens after exposure to T-2 toxin at a concentration of 13.5 mg/kg feed for 17 days. The same effects were found by Frankic et al. (2006) even at a lower concentration of 10 mg/kg T-2 exposure for 17 days.

However, Awad et al. (2012) demonstrated that the genotoxic effects of DON in broilers are not correlated with the induction of oxidative stress in the liver. In contrast, Borutova et al. (2008) reported induced oxidative stress in the liver of broilers fed with diets contaminated with DON and ZEA.

3.3. Aflatoxins

Aflatoxins as causing agents of the “turkey X-disease” in South-east England were responsible for the death of more than 100 thousand turkey poults in 1960. A shipment of peanut meal from Brazilian import was infected by *Aspergillus flavus* (Weidenbörner, 2001). The name ‘aflatoxin’ is derived from the "a" from *Aspergillus* and the "fla" from *flavus*. Not just *A. flavus*, but several strains of *A. parasiticus* (which predominates in warmer climate), *A. pseudotamari*, *A. bombycis* and *A. nominus* can also produce aflatoxins (Kurtzman et al., 1987).

This group of mycotoxins can be found mainly in tropical region with high temperature and humidity (Devegowda et al., 1998), but nowadays, there are increasing numbers of reports, that they can be produced even in temperate and continental climate regions (Giorni et al., 2007, Tabuc et al., 2009, Varga et al., 2010).

Preharvest infection of *A. flavus* and subsequent AFB_1 contamination occur in case of peanuts and maize. Drought stress of plants, drought enhanced insect damage, and wet weather conditions in combination with high temperatures, and cultivate of susceptible genotypes are important factors that promote invasion of this classically storage fungi in field (Weidenbörner, 2001, Szabó et al, 2015).

Aflatoxins are polycyclic, unsaturated, highly substituted coumarins. An aflatoxin molecule has two reactive sites, the unsaturated terminal site in the dihydrofurane moiety and the lactone ring of the coumarin part.

Although approximately 20 aflatoxins have been described, only four metabolites occur naturally in different feedstuffs, which were named according to their fluorescence at thin layer chromatography: blue, like aflatoxin B_1 (AFB_1) and aflatoxin B_2 (AFB_2) or green, like aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2).

The natural synthesis of AFB_1 is as follows: acetate – norslorinic acid – averantin – averufanin – averufin versiconal hemicacetal acetate – versicolorin A – sterigmatocystin – O-methylsterigmatocystin – AFB_1 (Weidenbörner, 2001).

AFB_1 is considered as being the most toxic mycotoxin (Kanora and Maes, 2009), and all of its metabolites show carcinogenic and mutagenic action (CAST, 2003). Aflatoxins may cause carcinogenicity, mutagenicty, teratogenicity and immunosuppression (Aycicek et al., 2005), and cause production losses in farm animals.

Aflatoxin M_1 and M_2, are the 4-hydroxylated derivates of AFB_1 and AFB_2, respectively. Mainly they can be found in milk, urine or egg white of animals that have ingested AFB_1 or AFB_2-contaminated feed (Weidenbörner, 2001).

A. flavus produces only AFB, while the other *Aspergillus* species produce both types of aflatoxins, B and G (Aycicek et al., 2005).

Aflatoxins are carcinogenic compounds as classified by the International Agency for Research in Cancer (IARC) in Group 1 with sufficient evidence of carcinogenicity in humans (IARC 2012).

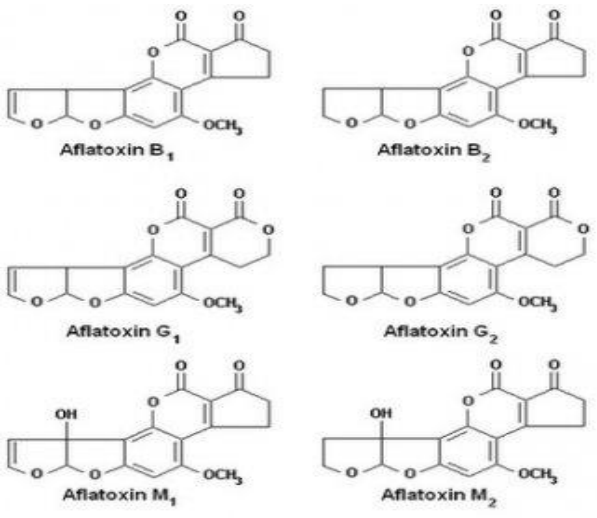
AFB_1 is the strongest natural carcinogen and the main hepatocarcinogen in animals, although its effects vary with species, age, sex, and nutritional status. Ducklings and pigs are very susceptible, while ruminants are more resistant (Weidenbörner, 2001). AFM_1 is also found in tissues and urine of affected animals and is also probably carcinogenic to humans (IARC Group 2A).

AFB_2 and AFG_2, and also AFM_2 are less toxic than their corresponding metabolites, due to the lack of the double bond terminating the difuran ring system, which is common in AFB_1, AFG_1 and AFM_1 (Weidenbörner, 2001).

Aflatoxin B_1 can be found in various feed- and foodstuffs. Mostly all agricultural crops like cereals (wheat, rice, maize, barley, oat and rye), nuts (hazelnut, peanut), dried fruits (e.g. fig, date) and spices are susceptible for contamination with aflatoxin (Diener et al., 1987).

The structures of the main aflatoxins and its metabolites are illustrated in Figure 3.

Figure 3. Chemical structure of the main aflatoxins (Goto and Marabe, 1989)



3.3.1. Metabolism and mode of action

AFB_1 is converted by cytochrome P450 enzymes (phase I xenobiotic transformation) to the reactive and electrophilic AFB_1-8,9-epoxide form, which is responsible for carcinogenic and toxic activity (Rawal et al. 2010).

In poultry species CYP2A6, CYP3A37, CYP1A5, and to a lower extent CYP1A1 orthologs are responsible for the bio-activation of AFB_1 (Dohnal et al., 2014). The epoxide metabolite can bind to guanine residues of DNA (causing genotoxicity) (Doi et al., 2002) and to proteins (causing cytotoxicity). Moreover, AFB_1-DNA adducts may result in guanine-cytosine (GC) to thymine-adenine (TA) transversions (Bennett and Klich, 2003). This leads to irreversible DNA damage, and causes hepatocellular carcinoma (Eaton and Gallagher, 1994).

The toxic epoxide metabolite may be detoxified in phase II xenobiotic transformation with gluthatione conjugation by glutathione-S-transferase (GST) (Dohnal et al., 2014), or hydrolysis by an epoxide hydrolase to AFB_1-8,9-dihydrodiol (AFB_1-dhd) or by metabolization to less toxic compounds such as aflatoxin M_1 (AFM_1) or aflatoxin Q_1 (AFQ1) (Diaz et al., 2010b).

AFM_1 is the main metabolite formed in the rumen of cattle and is excreted in milk, or in laying hens formed in the liver and excreted through the eggs, in egg white (Wolzak et al., 1985). This metabolite still has carcinogenetic properties (approx. 10 times lower than AFB_1).

Lozano and Diaz (2006) evaluated differences in hepatic *in vitro* metabolism of AFB_1 in different avian species. Microsomal and cytosolic liver fractions were obtained from both males and females of chicken, duck, quail and turkey. Males produced more AFB_1-8,9-epoxide than females, but significant differences between genders were observed only in ducks and turkeys. The cytosolic fraction from all four-species produced aflatoxicol (AFL). Turkey and duck hepatic cytosol produced more AFL than that of quail and chicken. AFL has about 18 times lower toxicity compared to AFB1 (Karabulut et al., 2014).

Age is another important factor affecting resistance of species to AFB_1. According to the results of Klein et al. (2002), younger poultry (turkey) are more sensitive to AFB_1 than older ones. This might be due to the lower activity of hepatic glutathione-S-transferase (GST) in young birds.

3.3.2. Toxicity of AFB_1 in poultry

A meta-analysis for broilers done by Andretta et al. (2011) encompassed 98 published articles, consisting of over 1,400 diets and 37,000 birds. This analysis showed that, aflatoxins reduced the feed intake and weight gain of broilers by 11%, and parallel increase in mortality (2.8 times higher). Effects were the greatest in young animals and, unsurprisingly, the mycotoxin concentration influenced the magnitude of the effect.

AFB_1 cause reduced growth rate and performance in poultry species, as it is described by several authors in broiler chickens: Giambrone et al. (1985b); Quezada et al. (2000), laying hens: Pandey et al. (2007), and turkeys: Giambrone et al. (1985a, b), or ducks: Chen et al. (2014).

AFB_1 also causes reduced feed intake of broiler chickens (Verma et al., 2004) and laying hens (Lee et al., 2012) and causes poor feed efficiency (Giambrone et al., 1985a, b).

In laying hens AFB_1 has caused reduced egg production and egg weight (Azzam et al.,1998; Khan et al., 2014), alterred egg quality parameters (Oliveira et al., 2003), increased liver fat content, changed organ weights, reduced protein levels in blood serum, carcass bruising, poor pigmentation and liver damage, decreased activities of several enzymes involved in the digestion of nutrients (e.g. starch, protein, lipids) (Devegowda and Murthy, 2005).

AFB_1 causes immunosuppression, resulting disease outbreaks, vaccination failures, and poor antibody titers (Devegowda and Murthy, 2005). The possible reason of immunosuppression caused by aflatoxins is their reactivity with T-cells. They also decrease phagocytic activity of macrophages (Dhanasekaran et al., 2001).

As a result of aflatoxicosis, liver is usually pale and enlarged, and also the gall bladder will become swollen (Dhanasekaran et al., 2001). Histologically, liver lesions include congestion of the hepatic sinusoids, focal hemorrhages, centrilobular fatty cytoplasmic vacuolation and/or necrosis, biliary hyperplasia, and nodular lymphoid infiltration (Leeson et al., 1995).

Osborne et al. (1982) described malabsorption syndrome characterized by steatorrhea, hypocarotenoidemia, and decreased concentrations of bile salts and pancreatic lipase, trypsin, amylase, and RNase in broiler chickens in case of AFB_1 toxicosis.

Yarru et al. (2009a) reported decreased hepatic gene expression of superoxide dismutase (SOD), glutathione S-transferase (GST), and epoxide hydrolase in chicks fed with 1.0 mg/kg AFB_1. In chickens fed with 2.0 mg/kg AFB_1, various hepatic genes associated with energy production and fatty acid metabolism (such as carnitine palmitoleyl transferase), growth and development (insulin-like growth factor 1), antioxidant protection (glutathione S-transferase), detoxification (epoxide hydrolase), coagulation (coagulation factors IX and X), and immune protection (interleukins) were downregulated, whereas genes associated with cell proliferation (ornithine decarboxylase) were upregulated (Yarru et al., 2009b).

The oral LD_{50} of AFB_1 in chick embryo is 0.025 mg/kg BW, while in day-old ducklings it is 0.3 mg/kg BW. In chicken, LD_{50} values vary between 2.0-6.3 mg/kg BW in different genotypes (Dhanasekaran et al., 2001).

3.4. European Commission regulations on maximum levels of mycotoxins in animal feed

There is contamination of feed and foodstuff of mycotoxin all over the world, therefore it is important to introduce a maximum level of concentration, but the actual level in different countries is different (van Egmond et al., 2007). European Commission (EC) Regulations and Recommendations has set an allowed concertation of mycotoxins in feed- or foodstuff for the betterment of human and animal welfare.

Maximum level depends on several factors, such as the species susceptibility and mycotoxin. For instance, several studies revealed that pigs are more susceptible for mycotoxin, like OTA, ZEN and DON than other farm animals. For that reason, maximum proposed level in feed of pigs is lower than

in poultry. Acute and chronic toxicity responsible for the negative effects of mycotoxins and the mechanism of their toxicosis is also different. AFB_1 is much more toxic, mutagenic and carcinogenic in chronic than in subchronic or acute exposure. Toxicokinetic and toxicodynamic value of a particular mycotoxin are important parameters for the undesirable action (Martinez-Larranaga at al., 2007).

Maximum permitted level of a particular mycotoxin is given both for complete or complementary feed, and also for the main feed ingredients, like cereals and maize. In nutshell, the maximum levels of the mycotoxin are usually higher in feed ingredients than in a complete feed.

The European Food Safety Authority (EFSA) has carried out risk assessments on several mycotoxins in animal feed that are considered to pose a potential risk on animal or human health: AFB_1, DON, ZEA, OTA, fumonisins and T-2/HT-2 toxin. In each case, EFSA has published an opinion that provides an assessment of the potential risk to animal or human health. Each opinion has been used as a basis for the current legislative controls on these mycotoxins (EFSA, 2014).

The presence of undesirable substances (chemical contaminants) in feed is controlled by European Community (EC) Directive 2002/32. The Directive sets maximum permitted levels for substances that are present in, or on, animal feed that pose a potential danger to animal or human health or to the environment or could adversely affect livestock production.

Currently, aflatoxin B_1 is the only mycotoxin with maximum permitted levels. These levels of AFB_1 have been set as low as reasonably achievable in order to protect animal and public health. The current limits for AFB_1 complete feeds (with a moisture content of 12%) for poultry (except young animals) is 0.02 mg/kg.

For the other mycotoxins guidance values have been recommended for feedstuffs and complete feeds. In case of DON the Commission Recommendation 2006/576/EC set a limit of 5 mg/kg for complementary and complete feedingstuffs for poultry (with a moisture content of 12%), while for the sum of T-2 toxin and HT-2 toxin the Recommendation 2013/165/EU set a limit of 0.25 mg/kg in complete feeds for poultry.

Correct evaluation of mycotoxin contamination is important in determination of the acceptable food and feed safety standard for mycotoxins. It is important, because distribution of mycotoxins is more often heterogeneous in feed or food commodities, refer as ‘hot-spots’, than the actual value of a representative sample. Correct sampling is the most critical point to determine the actual average concentration (Chaytor et al., 2011).

3.5. Formation, influence and effects of oxidative stress

Free radicals are molecules, atoms or ions having an unpaired electron in the outer orbital (Gilbert, 2000). Reactive oxygen species (ROS) can be formed in aerobic organisms as a result of some enzymatic processes, such as the mitochondrial respiratory chain (Murphy, 2009), or the oxidative burst mechanism of granulocytes (Slauch, 2011). They can also be formed in the immune system during normal physiological processes, for example, activation of nitrogen oxide (Bredt et al., 1990).

Superoxide radical ($O_2^{\bullet -}$), hydroxyl radical (OH•), hydroperoxyl radical (HO_2•), alkoxyl (RO•), and peroxyl radical (RO_2•) are examples for the ROS, while nitric oxide (NO•) and nitrogen dioxide (NO_2•) belong to the group of reactive nitrogen species (Dhawan, 2014).

There are also other reactive compounds, which are not free radicals according to the definition, but are generated by free radicals, and have important physiological functions or may cause detrimental effects in the living organism. Such non-radical reactive species are hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl) and hypobromous acid (HOBr) (Dhawan, 2014).

Reactive oxygen species are chemically reactive and can damage all of the biological macromolecules but play an important role in certain signaling pathways (Ignarro et al., 1999), in controlling cellular processes, or in the formation of non-specific immune response or induction of apoptosis (Engel et al., 2006). Thus, free radicals are “Janus-faced molecules” in biology, representing either beneficial or toxic effects. They serve as signaling and regulatory molecules at physiologic levels, but as highly deleterious and cytotoxic oxidants at pathologic levels (Fridovich, 1999).

ROS formation is controlled by the biological antioxidant defense system in physiological conditions, but if the balance of ROS formation and antioxidant defense system is impaired, oxidative stress develops (Sies, 1991). Disintegrating balance may be caused by increased ROS formation or decreased antioxidant protection (e.g. lack of fat or water-soluble vitamins, such as vitamin E, A or C; methionine/cysteine deficiency, lack of selenium, reduced activity of antioxidant enzymes) (Espinosa-Diez et al., 2015). Oxidative stress can desintegrate all cellular biomolecules. As a consequence, different cell structures may become damaged, but primarily the membrane phospholipids are affected by lipid peroxidation (Halliwell and Whiteman, 2004). Peroxidation of lipids primarily affects integrity of membranes as they have high content of polyunsaturated fatty acids in the phospholipid bilayer, which are particularly sensitive to peroxidative damage (Ayala et al., 2014).

When free radicals and other reactive species (e. g. •OH, HOO•, $ONOO^-$) extract a hydrogen atom from an unsaturated fatty acid chain, a carbon-centered lipid radical (L•) is produced. This is followed by the addition of oxygen to L• to yield a lipid peroxyl radical (LOO•). LOO• further propagates the peroxidation chain reaction by abstracting a hydrogen atom from a nearby unsaturated fatty acid.

Due to the rearrangement of the double bonds, conjugated dienes (CD) and conjugated trienes (CT) are formed (Takagi et al., 1987), resulting changes in membrane fluidity and therefore integrity.

The resulting lipid hydroperoxide (LOOH) can easily decompose to form a lipid alkoxyl radical (LO•). This series of ROS-initiated lipid peroxidation reactions with the production of lipid peroxyl and alkoxyl radicals, collectively called chain propagation, occurs in cells. Also, lipid hydroperoxides are unstable and in the presence of transition metal ions, such as iron or copper, they can decompose to produce new free radicals and cytotoxic aldehydes.

The process is terminated by a final termination phase in which the fatty acids are cleaved and metastable end products, such as aldehydes are produced (e.g. MDA, 4-hydroxy-2-nonenal) (Esterbauer and Cheeseman, 1990). These reactive aldehydes may precipitate proteins and even produce DNA adducts, leading to the formation of point mutations (Ayala et al., 2014). As a result of lipid peroxidation processes, due to the oxidative degradation of the fatty acids, the membrane fluidity and integrity decreases and therefore, their permeability is increased (Valko et al., 2007), which ultimately results in cell death and lysis (Kim et al., 2008).

3.6. The oxidative stress mechanisms associated with aflatoxins, DON and T-2 toxin

3.6.1. Aflatoxins

Oxidative stress plays a major role in the toxic effects of aflatoxins, as demonstrated by *in vitro* studies (Liu and Wang, 2016; Wang et al., 2017) and *in vivo* experiments (Shi et al., 2015). As a result of ROS formation induced by AFB_1, DNA damage (Zhang et al., 2015) and mitochondrial lesions (Liu and Wang, 2016) were detected.

In case of oxidative stress, several cell-organelles e.g. peroxisomes, mitochondria and endoplasmic reticulum are affected by the overproduction of free radicals, which is mainly associated with lipid peroxidation (da Silva et al., 2018). Aflatoxins disconnect oxidative phosphorylation in mitochondria, reduce mitochondrial membrane potential and thus, induce mitochondrial permeability (Shi et al., 2015; Liu and Wang, 2016) and also induce the formation of superoxide radical (Douarre et al., 2012).

Increased mitochondrial permeability results uncontrolled release of Ca^{2+} from mitochondria, which consequently leads to osmotic swelling and rupture of the outer mitochondrial membrane (Douarre et al., 2012). These mitochondrial changes caused by oxidative stress may result in cell apoptosis due to the release of cytochrome-c, changes in Bcl2/Bax expression, activation of mitogen-activated protein kinases (MAPK) and caspase-3 (Anuradha et al., 2001; Farley et al., 2006).

In case of broiler chicken, AFB_1 caused severe damage in cardiomyocytes, inhibited the mitochondrial functions, enhanced the ROS formation and at least provoked apoptosis (Wang et al., 2017).

When broiler chicken was treated with 150-300 μg/kg AFB_1 for 21 days, apoptotic cells were found in liver, which was accompanied by high level of malondialdehyde (MDA), end-product of lipid peroxidation in liver and kidney of treated birds (Ozen et al., 2009).

Aflatoxin-iduced oxidative stress caused, besides inhibiting the activity of antioxidant enzymes, and decreasing the level of GSH, leading to increased lipid-peroxidation (Ma et al., 2015). Aflatoxins also have effect on the regulation of antioxidant mechanisms, through the nuclear factor erythroid 2–related factor 2 (Nrf2), which is the main transcription factor for the expression of genes encoding antioxidant enyzmes, such as SOD, GPx and CAT (Liu and Wang, 2016; Wang et al., 2017).

Through the up-regulation of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), and interleukines (IL-1α, IL-1β and IL-6) and NO expression, AFB_1-induced ROS generation also influences inflammatory responses (Ma et al., 2015).

In case of mammals, such as mice or rats, AFB_1 treatment caused significant decrease in the activity of enzymes which play major role in the antioxidant defence, e.g. succinate dehydrogenase, glucose-6-phosphatase (G6P), GPx, GR, CAT, SOD and GST (Adedara et al., 2010; Rastogi et al., 2001).

Reduced quantity of GSH was followed by elevated MDA and NO- levels in the liver and kidney of AFB_1-treated chicken (Karaman et al., 2010).

3.6.2. DON

DON causes ribotoxic stress, and at molecular level inducing the MAPK phosphorylation, thus promoting apoptosis, resulting in changes of the inflammatory response and decreasing the expression of cell adhesion proteins (da Silva et al., 2014, Pierron et al., 2016).

Toxicity of DON is associated with oxidative stress and ROS-production, which has been confirmed in *vivo* (Borutova et al., 2008; Osselaere et al., 2013) and *in vitro* (Zbynovska et al., 2013).

DON alters the efficiency of intracellular antioxidant defense system in the target tissues such as liver, kidney, lymphoid organs, intestine and blood/serum, as demonstrated by increase in the MDA concentration (Li et al., 2014), and by decrease in the GSH content and activity of SOD, CAT or GPx (Zbynovska et al., 2013).

Erdélyi et al. (2011) investigated the effect of feeding a diet contaminated with low level of DON (1.5 mg/kg feed) in broiler chickens. MDA concentration was significantly higher in liver homogenate on day 21 in the group fed with DON contaminated diet, but GSH content and GPx activity did not differ significantly between the treatment groups.

Several studies have shown that the oxidative stress is involved in the apoptosis, DNA fragmentation and cell death induced by DON (Frankic et al., 2008; Zhang et al., 2009, Wu et al., 2014), as well as the inhibition of protein synthesis and increase in protein carbonyl content, as a consequence of free radical-mediated protein oxidation (Strasser et al., 2013).

3.6.3. T-2 toxin

The main target of T-2 toxin, as a 'Type A' trichothecene, is the ribosome, affecting initiation of the polypeptide chain (Li et al., 2011). T-2 toxin inactivates peptidyl transferase activity resulting in inhibition of protein synthesis and disruption of the morphology of cell membranes and mitochondria (Adhikari et al., 2017).

In vitro (Yang et al., 2016; Zhang et al., 2016) and also *in vivo* studies (Chaudhari and Lakshmana, 2010) showed that T-2-induced oxidative stress is associated with an increase in ROS generation, therefore DNA and protein oxidation, and lipid peroxidation leading to apoptosis (Chaudhari et al., 2009, Wu et al., 2014).

The oxidative stress induced by T-2 toxin promotes Fas up-regulation, tumour suppressor protein p53 activation, down-regulation of Bcl-2 (B-cell lymphoma 2) and up-regulation of the pro-apoptotic factor Bax (apoptosis regulator BAX, also known as bcl-2-like protein 4), causing cytochrome-c release, caspase-3 activation and at last apoptosis (Zhang et al., 2018).

T-2 toxin, at high level of contamination, induces apoptosis signalling pathway through the activation of c-Jun N-terminal kinase 1 (JNK1), p38 MAPK, and increase in heat shock protein (Hsp) 70 expression, and activity of inducible nitric oxide synthase (iNOS) activity, also NO release, therefore causing mitochondrial damage and activation of caspase 3 (Chaudhari and Lakshmana, 2010; Li and Pestka, 2008). T-2 toxin can modulate the inflammatory response by increasing the expression of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β and IL-11) (Agrawal et al., 2012; Zhou et al., 2014).

ROS generation, depends on this value, causes increase or decrease in Nrf2 expression, consequently increase or decrease the expression of genes encoding antioxidant enzymes, such as GPx, GR, SOD and CAT, and promoting an increase or decrease in GSH level through higher or lower activity of glutathione synthetising enzymes, thus decreasre or increase lipid peroxidation, as shown by lower or higher MDA level (Wu et al., 2014; Yang et al., 2016).

Balogh et al. (2015) found that T-2 toxin (1.5 or 3.4 mg/kg feed) exposure-initiated lipid peroxidation and activated the glutathione redox system in a four-week-long experiment done with broiler chicken, but the changes were irrespective of the dose- and partly duration of the exposure. Bócsai et al. (2015) investigated the short-term effects of oral T-2 toxin exposition (5, 10 or 15 mg T-2 toxin/kg feed) on oxidative stress parameters in different tissues of laying hens. GSH concentration and GPx activity did not show time- and dose-dependent changes in blood plasma. Conjugated diene and triene levels were significantly higher at the end of the trial in the 15 mg T-2 toxin/kg feed treatment in liver, however MDA concentration did not change. GSH concentration and GPx activity were significantly higher at the highest dose T-2 toxin in liver homogenates, but not in spleen or kidney.

3.7. Biological antioxidant system

During the evolution, to evoke oxidative effects, antioxidant defense systems have evolved in the living organisms, which have the function of protecting the organism from damage caused by ROS (Sies, 1997). In higher-order organisms, the antioxidant system is based on the three major levels of defence:

1. The first line of protection is based on small molecular weight antioxidants. These are molecules with nucleophilic and reducing properties, capable of reacting with ROS, acting as chain-breaking antioxidants, as electron donors. They can be fat-soluble (eg. vitamins A and E) or water-soluble (e.g. vitamin C, reduced glutathione, uric acid) molecules. Antioxidant molecules breaking the free radical chain reaction, while for an extremely short period of time and at low rate they are also transformed to free radicals, after which they are reduced promptly with great efficiency. Fat-soluble α-tocopherol is physically linked to membrane phospholipids; thus it is directly protecting them from oxidative damage. After oxidation, it is converted to tocopheryl quinone which is less hydrophobic and therefore partially cleavages from the membrane phospholipid bilayer. Oxidized α-tocopherol (tocopheryl-quinone) is primarily reduced by L-ascorbic acid in the cytosol, which becomes hydrophobic so that it can contact the membrane phospholipids again (Tokarz et al., 2013). Reduced glutathione (GSH), a water-soluble tripeptide is also a key component of non-enzymatic antioxidant protection. Two enzymes are involved in the synthesis of GSH, γ-glutamyl-cysteine synthetase (GCS) catalyzing glutamate and cysteine linkage, and glutathione synthase (GS) which links a glycine amino acid to the dipeptide in an ATP-dependent process (Espinosa-Diez et al., 2015).

In the cells glutathione, is present in reduced form as an active thiol, which is easily oxidized to glutathiyl radical (GS•) during oxidative stress, so it is an important component of protection against oxidative processes (Volodymyr, 2011). Two glutathiyl radicals dimerize to form glutathione disulfide (GSSG). GSH is capable of eliminating ROS directly and indirectly. On the one hand, it can directly react with the superoxide anion and other free radicals, and indirectly, it can reduce other antioxidant molecules such as dehydro-ascorbic acid (Espinosa-Diez et al., 2015). GSH homeostasis is not only influenced by *de novo* synthesis but also by other factors, such as intracellular use, recycling (GSSG ↔ GSH) catalyzed by glutathione reductase (GR), or the rate of efflux from the cell. GSH also plays a role in protecting the body's enzymatic antioxidant system as co-substrate of glutathione-peroxidases (GPx) or glutathione-S-transferases (GST).

2. The second line of defense is based on antioxidant enzymes (SOD, CAT, GST, GPx, thioredoxin reductases) and some metal binding chelating proteins (e.g. ferritin, metallothionein) (Valko et al., 2007). Antioxidant enzymes neutralize reactive oxygen radicals (Valko et al., 2007), and chelating proteins act to bind transitional metals (e.g. Fe, Cu) that may induce ROS formation in Fenton-type reactions (Birben et al., 2012).

Superoxide anions that are continuously formed in mitochondria and microsomes are neutralized by superoxide dismutases (SOD). The ancient type Mn-SOD is found in prokaryotes and mitochondria of eukaryotes. In the cytosol of eukaryotic cells Cu-Zn SOD is present (Espinosa-Diez et al., 2015). Almost complete inventory of catalase is found in microsomes, nucleus and peroxisomes (Droge, 2002). GPx superfamily also plays an important role in the control of harmful effects of ROS,

primarily in cytosol and mitochondria, but also in membranes, as reducing harmful peroxides (hydrogen peroxide or organic peroxides) to form their corresponding alcohols or water, using GSH as an electron donor co-substrate (Erdélyi et al., 1999).

The glutathione S-transferase enzyme family is not only antioxidant, because during the phase II xenobiotic transformation processes these enzymes catalyze the biosynthetic conjugation of GSH and electrophilic xenobiotics (Hayes and Strange, 1995). They have similar activity to GPx, mainly reducing the organic hydroperoxide using GSH as a co-substrate, mainly in selenium-deficient conditions (Pickett and Lu, 1989).

3. In the third line of antioxidant defense, repair molecules and enzymes, e.g. heat shock proteins (Hsp), certain lipases, proteases, and DNA repair enzymes and glutathione reductase (GR) catalyze the recovery and/or removal of damaged/oxidized macromolecules (Davies, 2000).

3.8. Phytobiotics

Phytobiotics are herb-derived substances having beneficial effect on performance and/or health status of livestock due to their complex biologically active components. The primary mode of action of phytogenic feed additives is to stabilize feed hygiene and even more to beneficially affecting the ecosystem of gastrointestinal microbiota by controlling potential pathogens (Roth and Kirchgessner, 1998).
Kumar et al. (2014) classified the beneficial effects of feed additives into six categories, which is also true for the phytobiotics:

- influence on feed intake, digestibility of nutrients and animal performance
- antimicrobial effects
- anti-inflammatory effects
- antioxidant effects
- stimulating effects on immune response
- coccidiostatic effects.

These additives might be composed of certain organs (leaf, bark, seed, fruit etc.) of the medicinal plants, or – what is even more common nowadays – we use their essential oils or extracts having high concentration of biologically active molecules. Specific uses of plants have varied in accordance to parts utilized (Table 1).

Table 1. Different herbal feed additives, active components and functions (Mirzaei-Aghsaghali, 2012)

Plant	Used parts	Active component	Function
Nutmeg (*Myristica fragans*)	Seed	Sabiene	Digestion stimulant, antidiarrhoeic
Cinnamon (*Cinnamomum zeylanicum*)	Bark	Cimetaldehyde	Appetite and digestion stimulant, antiseptic
Cloves (S*yzygium aromaticum*)	Cloves	Eugenol	Appetite and digestion stimulant, antiseptic
Cardamom (*Amomum subulatum*)	Seed	Cineol	Appetite and digestion stimulant
Coriander (*Coriandrum sativum*)	Leaves and seed	Linalool	Digestion stimulant
Cumin (*Cuminum cyminum*)	Seed	Cuminaldehyde	Digestive, carminative, galactogogue
Anise (*Pimpinella anisum*)	Fruit	Anethol	Digestive, carminative, galactogogue
Celery *(Apium graveolens*)	Fruit, leaves	Phtalides	Appetite and digestion stimulant
Parsley (*Petroselinum crispum*)	Leaves	Apiol	Appetite and digestion stimulant, antiseptic
Fenugreek (*Trigonella foenum-graecum*)	Seed	Trigonelline	Appetite stimulant
Capscicum (*Capsicum annum*)	Fruit	Capsaicin	Digestion stimulant
Pepper (*Piper nigrum*)	Fruit	Piperine	Digestion stimulant
Horseradish (*Armoracia rusticana*)	Root	Allyl izotiocianat	Appetite stimulant
Mustard (*Brassica nigra*)	Seed	Allyl izotiocianat	Digestion stimulant
Ginger (*Zingiber officinale*)	Rizom	Zingerone	Gastric stimulant
Garlic (*Allium sativum*)	Bulb	Alkin	Digestion stimulant, antiseptic
Rosemary (*Rosmarinus officinalis*)	Leaves	Cineol	Digestion stimulant, antiseptic
Thyme (*Thymus vulgaris*)	Whole plant	Thymol	Digestion stimulant, antiseptic, antioxidant
Mint (*Mentha piperita*)	Leaves	Menthol	Appetite and digestion stimulant, antiseptic
Shatavari (*Asparagus racemosus*)	Root	Sapogenins, flavonoids and saponin	Prevention and treatment of gastric ulcers, dyspepsia and as a galactogogue
Jivanti *(Leptadenia reticulata*)	Leaves and twigs	Stigmasterol, beta-itosterol, flavonoids, pregnane glycosides	Galactogogue, antimicrobial and anti-inflammatory activity
Shatavari (*Asparagus racemosus*)	Root	Shatavarin-I-IV, rutin, hyperoside	Galactagogue

Herbal compounds might contain various active components such as polyphenols, flavonoids and organosulfur molecules, consequently their biological activity is highly variable. Some of them are digestion stimulants, others have antioxidant or bacteriostatic activity. The latter property results in stabilizing of the microbiota, which is the most common effect confirmed in the literature (Máthé, 2009). Majority of herbs have numerous active components and their biological effect is attributable

to the dominant one. However, often the realized effect is a result of synergism and/or antagonism among the different active substances (Wald, 2004).

In most of the commercially available phytobiotic products blends of herb extracts are used, which makes the predictability of final effect even more uncertain. So, when herbs are used in combination, the effects can be complicated as various interactions can occur even among the individual dominant components (Che et al., 2013).

Phytobiotic products might be good alternatives of the antibiotic growth promoters, which are banned to use in many countries (Mountzouris et al., 2009). There is huge demand for natural additives because of having great potential to replace the antioxidant agents and synthetic antimicrobial agents with natural ones (Rasooli et al., 2006). In a recent study, Ahmed et al. (2014) have shown that herbs are also capable to increase shelf life of feed and food, which is based on their inhibitory effect on various harmful microorganisms.

3.8.1. Phytobiotics against mycotoxins

Mycotoxins – as it was discussed before - are one of the most potent feed safety issues in livestock production nowadays. To prevent their harmful effects there are several more or less effective additives, in particular when high concentration mycotoxin contamination occurs in feedstuffs. However, mycotoxins are frequently present in the feedstuffs at low concentration, causing only nonspecific symptoms or subclinical effects. The most commonly realized problem is depression of the production traits. Herb extracts might have good potential in such cases to minimize losses caused by these fungal toxins.

There are several results in the literature on the positive effects of phytogenics over aflatoxins in the diet of poultry. Thus, Manafi et al. (2014a) studied the effect of rosemary (500 mg/kg) on aflatoxin B_1 (600 μg/kg) in broiler chicken and found beneficial results in the performance traits. Similar results were reported by Diaz et al. (2009) in turkey. Ethanolic turmeric extract (0.05%) is also a good candidate to prevent negative effects of aflatoxin (3 mg/kg) on performance of broilers according to Rangsaz and Ahangaran (2011). Licorice extract (LE; 150 mg/kg) was found to be highly beneficial on carcass traits and broilers chicken's performance, when 2 mg/kg aflatoxin was present in the diet (Al-Daraji, 2012).

Aflatoxins have histopathological effects and the target organs are liver and kidney. Turmeric extract (*Curcuma longa*) revealed to be efficient to protect liver and kidney against toxic effects of aflatoxins in chicken (Gholami-Ahangaran et al., 2015). In a similar experiment Abd El-Ghany et al. (2013) found that adding turmeric powder (80 mg/kg) in the diet with aflatoxin contamination (2.5 mg/kg) can prevent its pathological lesions in the liver and kidney. Similar hepatoprotective effects were found by Karna (2013) with *N. sativa* seed (2%) in aflatoxicosis.

Research of Kumar et al. (2013) has confirmed that introducing citrus fruit oil (2.5 g/kg) in aflatoxin contaminated (1 mg/kg) broiler chicken diet can moderately prevent bile duct hyperplasia and hydropic degeneration in liver caused by aflatoxin.

Phytogenics might have potentials to reduce immune suppressive effects of mycotoxins. Sadeghi et al. (2014) have included savory essential oil (500 mg/kg/dry matter) into aflatoxin contaminated (0.5 mg/kg) broiler diet for 42 days which minimized the effect of aflatoxin on humoral immune responses of treated chickens. Increased antibody titers were measured due to thyme oil treatment in aflatoxicosis in broiler chicken (Manafi et al., 2014b).

Aflatoxin (1 mg/kg) caused decreased antioxidant functions in terms of level of peroxides, SOD activity, and total antioxidant concentration in liver were found to be also alleviated by turmeric powder (Gowda et al., 2008) and by its ethanol extract (Rangsaz and Ahangaran, 2011).

Application of some herbal extracts, like turmeric (*Curcuma longa*), garlic (*Allium sativum*) and asafetida (*Ferula asafetida*) have shown to counteract aflatoxicosis in poultry through their antioxidant activity, which might be caused by antioxidant substances capable of scavenging free radicals and/or enhancing antioxidant enzymes. The most of these inhibitors are chemically phenylpropanoids, terpenoids and alkaloids (Holmes et al., 2008).

Other oils, like peppermint, basil, cinnamon also show beneficial effects in case of feeding aflatoxin infected corn, and those phenolic agents might help to reduce the effect of mycotoxin (Dvorska et al., 2007).

Abdelhamid et al. (2002) showed that various herbs and medicinal plants reduced the toxicity of different mycotoxins. Some seeds, such as fenugreek seed, basil seed and roquette seed, black seed, and some herbs, like liquorice, garlic, onion and coriander also counteract the effect of mycotoxins (Salem et al., 2010). Thyme oil also showed inhibitory effect on different varieties of mycotoxin producing fungi (Couladis et al., 2004). A detailed study with thyme oil active compounds showed that it has measurable inhibitory effect on mycotoxins (Rasooli, 2004).

Though research data on interaction between phytogenics and mycotoxins are related mainly to aflatoxins. However, there are some previous results on beneficial effect of garlic extract in broiler chicken upon T-2 toxin intoxication (Ancsin et al., 2013).

To achieve the beneficial effect of different active components from (medicinal) plants and herbs, manufacturers often produce a mixture of them. Herbamix Basic Premix – the product used in my experiments – contains essential oils from 7 plants and glycerol extract of two plants. The active compounds (e.g. allyl cysteine, alliin, allicin, and allyl disulfide) of garlic (*Allium sativum*) have antioxidant properties (Chung, 2006), while Ahmad et al. (2011) found positive effects of rosemary (*Rosmarinus officinalis*) extract (e.g. phenolic diterpens such as carnosic acid, carnosol, rosemanol, epi- and isorosmanol, rosmadial and methyl carnosate) on immune responses and oxidative stress in case of aflatoxin exposure in mice. Abdelrazek et al. (2015) described ameliorative effects of the main active compounds of oregano (*Origanum vulgare*), namely carvacrol and thymol, on aflatoxin-induced immune impairments in growing Japanese quail, while Ri et al. (2017) reported beneficial effects of dietary oregano powder supplementation on antioxidant status of broiler chickens. In an experiment done with Ross 308 broiler chickens Faix et al. (2009) described significant antioxidant properties of eugenol and cinnamic aldehyde from true cinnamon tree (*Cinnamomum verum*). The main active compounds of common thyme (*Thymus vulgaris*), such as thymol, carvacrol and borneol

also have antioxidant properties, and can reduce the aflatoxin-induced oxidative stress as it was found in rats by El-Nekeety et al. (2011). The oil extracted from the leaves of narrow-leaved paperbark (*Melaleuca alternifolia*) contains over hundred bioactive compounds, with terpinen-4-ol and 1,8-cineole as the major components, which are responsible for its antioxidant and antimicrobial properties (Zhang et al., 2018). Eucalyptol, the main component in the essential oil of southern blue-gum (*Eucalyptus globulus*) has antifungal and antimycotoxigenic activity (López-Meneses et al., 2015). According to the results of Saeed et al. (2018) the chicoric acid in the glycerol extract of eastern purple coneflower (*Echinacea purpurea*), and silimarin in the glycerol extract of Mediterranean milk thistle (*Silybum marianum*) have immunostimulant and growth promoting effects in the presence of immunosuppressant aflatoxin B1 in the broiler feed (Chand et al., 2011).

4. MATERIALS AND METHODS

4.1. Experiments investigating the short-term effect of herbal mixture against the detrimental effects of high mycotoxin exposure

4.1.1. Investigation of the effects of a medicinal herb mixture and DON or T-2 toxin exposure in broiler chickens

The experiment done with day-old Cobb 540 chickens (n=150, average body weight: 50 g) were obtained from Babádi Baromfikeltető Ltd. (Ócsa-Felsőbabád, Hungary). The trial was carried out at the Experimental Farm of Department of Nutrition, Szent István University. The birds were reared on pine shavings bedding for 19 days. At 19th day of life the birds were grouped, and the experiment started after 2 days of adaptation period. At grouping all birds were weighed individually to compose similar groups, therefore the difference between the average body weights in the groups did not exceed 5%. Each experimental group contained 12 broilers in two replicates. Feed (broiler intensive grower complete diet, Vitafort Ltd., Dabas, Hungary) and water were provided *ad libitum*. The complete feed was free of coccidiostats and toxin-binders, and its nutrient content was as follows: crude protein: 19.34%, crude fiber: 4.10%, crude fat: 2.90%, crude ash 7.40% ME: 10.69 MJ/kg, Vitamin A: 10050 IU/kg, Vit. D_3: 3015 IU/kg, Lys: 0.95%, Met: 0.45%. The nutrient content of the diet met the requirements of broiler chickens (Hungarian Feed Code, 2004). The cause of the relatively low energy content is that the manufacturer offers this coccidiostat and toxin-binder free diet to small holders.

For the measurement of transit time of the feed particles in the gastronintestinal tract 6 birds were kept separately and fed methyl-orange dye coloured diet. Time lap between intake and excretion of the dye was measured to determine transit time.

4.1.1.1. Experimental design

A total of 144 three-week-old broiler chickens (body weight: 749.60±90.98 g) was randomly selected and divided into six groups of 24 birds each. Beside the control group, 5 treated groups were formed, as shown below in Table 2:

Table 2. The different treatments used in the broiler experiment

Groups	Diet	No. of birds
Control	intensive grower diet	12x2
Treated I. (**T-2 toxin treated [T]**)	intensive grower diet + [T-2 toxin (3.72 mg/kg) and HT-2 toxin (1.26 mg/kg)]	12x2
Treated II. (**DON treated [D]**)	intensive grower diet + DON (16.12 mg/kg)	12x2
Treated III. (**Herbamix treated [H]**)	intensive grower diet +Herbamix Basic premix (600 mg/kg)	12x2
Treated IV. (**T-2 toxin + Herbamix treated [TH]**)	intensive grower diet + [T-2 toxin (3.72 mg/kg) and HT-2 toxin (1.26 mg/kg)] + Herbamix Basic premix (600 mg/kg)	12x2
Treated V. (**DON + Herbamix treated [DH]**)	control diet + DON (16.12 mg/kg) + Herbamix Basic premix (600 mg/kg)	12x2

The light program followed the guidelines for Cobb 540 broilers until the start of the experimental period, then continuous light regimen was used for the whole period (48 hours) of mycotoxin exposure.

4.1.1.2. Preparation of feed

4.1.1.2.1. Artificial mycotoxin contamination of the feed

T-2 toxin was produced by *Fusarium sporotrichioides* (NRRL 3299) and DON by *Fusarium graminearum* (NRRL 5883) strains on corn substrate according to Fodor et al. (2006). The corn substrates with known trichothecene mycotoxin concentrations, were mixed to broiler chicken feed in order to reach the final concentration of intended doses. Concentration of T-2/HT-2 toxin and DON was determined in control and mycotoxin contaminated feeds. T-2 and HT-2 toxin concentrations were measured based on the method of Trebstein et al. (2008) and DON content according to Pussemier et al. (2006) with HPLC method after immunoaffinity cleanup.

Measured mycotoxin concentrations of the commercial diet (1 kg) were: T-2 toxin: <0.10 mg; DON: 0.25 mg. The experimentally mycotoxin contaminated diets contained (1 kg) 3.74 mg T-2 toxin and 1.26 mg HT-2 toxin in groups Treated II and IV, or 16.12 mg DON in groups Treated III and V, respectively.

4.1.1.2.2. Preparation of herbal mixture containing feed

Herbal mixture (Herbamix Basic Premix™, Herbamix Trade Ltd., Budapest, Hungary) was added to the complete feed in powder form at the dose of 600 mg/kg. The Herbamix Basic Premix contains essential oils from 7 plants, namely garlic (*Allium sativum*), rosemary (*Rosmarinus officinalis*),

oregano (*Origanum vulgare*), true cinnamon tree (*Cinnamomum verum*), common thyme (*Thymus vulgaris*), narrow-leaved paperbark (*Melaleuca alternifolia*) and southern blue-gum (*Eucalyptus globulus*) standardized on their active substance. Glycerol extract of two plants, namely eastern purple coneflower (*Echinacea purpurea*) and Mediterranean milk thistle (*Silybum marianum*) were also components of the product.

4.1.1.3. Sampling

The short-term trial lasted for 2 days after 12 hours of feed deprivation. Six randomly chosen birds were exterminated from each group at 12^{th}, 24^{th}, 36^{th} and 48^{th} hours of the experiment. The first sampling started at 8 o'clock in the evening followed by 12 hours up to the end of the trial (48 hour).

After cervical dislocation, whole blood samples were collected into EDTA-Na_2 containing tubes. *Post mortem* liver and kidney samples were collected at every 12 hours.

The whole blood was separated by centrifugation (2,500g, 10 min) and blood plasma was collected. Red blood cell (RBC) hemolysates were prepared with 9-fold distilled water. Blood plasma, RBC hemolysate, liver and kidney samples were stored at −70 °C until the biochemical analyses. Before the analyses the samples were thawn at room temperature, and liver, kidney was homogenised with 9-fold cold (4 °C) physiological saline (0.65% w/v NaCl).

4.1.1.4. Weighing

At each sampling, before the extermination, live weight of the birds was measured. *Post mortem* the weight of liver samples was also recorded. From these data relative liver weights (g/100 g live weight) were calculated.

To calculate the feed consumption of the birds during the experiment the feeders were weighed at every 4 hours in each experimental group.

4.1.2 Investigation of the effects of a medicinal herb mixture and short-term aflatoxin toxin exposure in laying hens

The animal trial was carried out at the Experimental Farm of Department of Nutrition, Szent István University. Total of 60 Bovans Goldline laying hens being at 90% daily egg production at 49 weeks of age were used. The hens were kept on deep litter.

4.1.2.1. Experimental design

Two days before the experiment the laying hens were randomly selected and divided into four groups of 15 layers each. Beside the control, 3 treated groups were formed, as shown below in Table 3:

Table 3. The different treatments used in the experiment with with laying hens

Group	Treatment	No. of birds
Control [C]	Control layer diet	15
Treated I. (**Herbamix treated [H]**)	Control layer diet + Herbamix Basic premix (600 mg/kg)	15
Treated II. (**Aflatoxin treated [A]**)	Control layer diet + aflatoxin (125 µg AFB_1/kg)	15
Treated III. **(Aflatoxin + Herbamix treated (AH)**	Control layer diet + aflatoxin (125 µg AFB_1/kg) + Herbamix Basic premix (600 mg/kg)	15

The experiment lasted for 2 days, started after 2 days of adaptation period. During the trial restricted feeding was used (150 g per hen), but drinking water was provided *ad libitum*.

The laying hens were fed with „Golden" complete feed for layers, manufactured by Vitafort Ltd., Dabas, Hungary. The nutrient content of the feed was: crude protein: 17.36%, crude fiber: 4.90%, crude fat: 2.70%, crude ash 15.00%, ME: 9.86 MJ/kg, Vitamin A: 10120 IU/kg, Vit. D_3: 3542 IU/kg, Lys: 0.84%, Met: 0.43%, phytase: 500.0 FTU/kg.

This nutrient content of the diet met the requirements of laying hens (Hungarian Feed Code, 2004).

For the measurement of transit time of the feed particles in the gastrointestinal tract 5 laying hens were kept separately and fed methyl-orange dye coloured diet.

In case of laying hens light regimen of 12 hours daylight and 12 hours dark period was used.

4.1.2.2. Preparation of feed

4.1.2.2.1. Artificial mycotoxin contamination of the feed

Aflatoxin was produced by *Aspergillus flavus* strain (ZT80) isolated by Dobolyi et al. (2011), on ground corn substrate. The corn substrate, with known aflatoxin concentration was mixed to the feed in order to reach the final concentration of intended doses.

Aflatoxin content of the loaded complete feed was analyzed with AFLAPREP HPLC method after immunoaffinity clean-up (Food Analytica Ltd., Gyula, Hungary).

Measured total aflatoxin concentration of the commercial diet was <1.0 µg/kg. Measured total aflatoxin concentration of the aflatoxin contaminated feed was: 170.3 µg/kg, in which AFB_1: 125 µg/kg; AFB_2: 39.0 µg/kg; AFG_1: 2.0 µg/kg; AFG_2: 4.3 µg/kg.

4.1.2.2.2. Preparation of herbal mixture containing feed

Herbal mixture (Herbamix Basic Premix™, Herbamix Trade Ltd., Budapest, Hungary) was added to the complete feed (to control and to aflatoxin contaminated) in powder form at the dose of 600 mg/kg.

4.1.2.3. Sampling

The sampling protocol was identical as it is written in chapter 4.1.1.3.

4.1.2.4. Weighing

At every sampling, before extermination live weight of the hens was measured. *Post mortem* the weight of liver samples was also recorded. From these data relative liver weights (g/100 g live weight) were calculated.

To calculate the feed consumption of the birds during the experiment, the feeders of each experimental group were weighed at every 4th hour.

4.2. Effect of short term T-2 toxin or aflatoxin exposure in broiler chickens with medicinal herb mixture pre-treatment against the detrimental effects of high mycotoxin exposure

4.2.1. Investigation of the effects of a medicinal herb mixture and T-2 toxin exposure in broiler chickens

4.2.1.1. Experimental design

Day-old Cobb 540 broiler chickens (n=210, average body weight: 50 g) were obtained from Babádi Baromfikeltető Ltd. (Ócsa-Felsőbabád, Hungary). The trial was carried out at the Experimental Farm of Department of Nutrition, Szent István University. The birds were divided into five groups of 42 chickens each. They were reared on pine shavings bedding. Feed and water were provided *ad libitum*.

From the 1st day of age the broilers were fed with broiler grower complete feed free of coccidiostats and toxin-binders (Vitafort Ltd., Dabas, Hungary). The nutrient content of this feed was the following: crude protein: 19.34%, crude fiber: 4.10%, crude fat: 2.90%, crude ash: 7.40%, ME: 10.69 MJ/kg, Vitamin A: 10050 IU/kg, Vit. D_3: 3015 IU/kg, Lys: 0.95%, Met: 0.45%.

Nutrient content of the diet met the requirements of broiler chickens (Hungarian Feed Code, 2004).

From the 7th day of age changes were made in the diet of the different groups, as it is shown below in Table 4:

Table 4. The different treatments used in broiler experiment with medicinal herb mixture pre-treatment

Groups	Diet	No. of birds
Control (C)	Intensive grower diet (from 7th day of age)	21x2
Control (C)*, ***** later: Treated I.	Intensive grower diet (from 7th until 21st day of age)	21x2
Treated II. (**With low dose of Herbamix treated [H1]**)	Intensive grower diet + Herbamix Basic Premix™ (300 mg/kg) from 7th day of age	21x2
Treated III. (**With medium dose of Herbamix treated [H2]**)	Intensive grower diet + Herbamix Basic Premix™ (600 mg/kg) from 7th day of age	21x2
Treated IV. (**With high dose of Herbamix treated [H5]**)	Intensive grower diet + Herbamix Basic Premix™ (1500 mg/kg) from 7th day of age	21x2

From the 21st day of age the treated groups received T-2 toxin contaminated feed as shown below:

Groups	Diet	No. of birds
Control (C)	Intensive grower diet	12x2
Treated I. **(T-2 toxin treated [T])**	Intensive grower diet + T-2 toxin (1.13 mg/kg)	12x2
Treated II. (**With low dose of Herbamix + T-2 toxin treated [H1+T]**)	Intensive grower diet + Herbamix Basic Premix™ (300 mg/kg) + T-2 toxin (1.13 mg/kg)	12x2
Treated III. (**With medium dose of Herbamix + T-2 toxin treated [H2+T]**)	Intensive grower diet + Herbamix Basic Premix™ (600 mg/kg) +T-2 toxin (1.13 mg/kg)	12x2
Treated IV. (**With high dose of Herbamix + T-2 toxin treated [H5+T]**)	Intensive grower diet + Herbamix Basic Premix™ (1500 mg/kg) + T-2 toxin (1.13 mg/kg)	12x2

The light program followed the guidelines for Cobb 540 broilers until the start of the experiment, then continuous light regimen was used for the whole period (48 hours) of mycotoxin exposure.

4.2.1.2. Preparation of feed

4.2.1.2.1. Preparation herbal mixture containing feed

Herbal mixture (Herbamix Basic Premix™, Herbamix Trade Ltd., Budapest) was added to the complete feed in powder form at 3 different doses:

- For the group fed with low dose of Herbamix, the premixture was added in 300 mg/kg concentration, which is equal to the supplier's recommendation.

- For the group fed with medium dose of Herbamix, the premixture was added in 600 mg/kg concentration, which is twice as high as supplier's recommendation.

- For the group fed with high dose of Herbamix, the premixture was added in 1500 mg/kg concentration, which is five times higher than the supplier's recommendation.

4.2.1.2.2. Artificial mycotoxin contamination of the feed

T-2 toxin was produced by *Fusarium sporotrichioides* (NRRL 3299) strains on corn substrate according to Fodor et al. (2006). The corn substrate, with known trichothecene mycotoxin concentrations was mixed to broiler chicken feed in order to reach the final concentration of intended doses.

Concentration T-2 toxin/HT-2 toxin was determined in control and mycotoxin contaminated feeds based on the method of Trebstein et al. (2008) with HPLC method after immunoaffinity cleanup.

Measured mycotoxin concentration of the commercial diet (1 kg) was: T-2 toxin: <0.10 mg; The experimentally mycotoxin contaminated diets contained (1 kg) 1.13 mg T-2 toxin and <0.10 mg HT-2 toxin.

4.2.1.3. Sampling and weighing

The short-term trial lasted for 2 days, after 12 hours of feed deprivation. Six randomly chosen birds were exterminated from each group at 12^{th}, 24^{th}, 36^{th} and 48^{th} hours of the experiment.

The sampling and weighing protocol was identical to the one shown earlier in chapters 4.1.1.3. and 4.1.1.4.

4.2.2. Investigation of the effects of a medicinal herb mixture and aflatoxin exposure in broiler chickens

4.2.2.1. Experimental design

The experiment was done with day-old Cobb 540 broiler chickens (n=210, average body weight: 50 g) were obtained from Babádi Baromfikeltető Ltd. (Ócsa-Felsőbabád, Hungary). The animal trial was carried out at the Experimental Farm of Department of Nutrition, Szent István University. The birds were divided into five groups of 24 chickens each. They were reared on pine shavings bedding. Feed and water were provided *ad libitum*.

From the 1st day of age the chickens were fed with broiler grower complete feed free of coccidiostats and toxin-binders (Vitafort Ltd., Dabas, Hungary). The nutrient content of this feed was the following: crude protein: 19.34%, crude fiber: 4.10%, crude fat: 2.90%, crude ash: 7.40%, ME: 10.69 MJ/kg, Vitamin A: 10050 IU/kg, Vit. D_3: 3015 IU/kg, Lys: 0.95%, Met: 0.45%.

Nutrient content of the diet met the requirements of broiler chickens (Hungarian Feed Code, 2004). The cause of the relatively low energy content is that the manufacturer offers this coccidiostat and toxin-binder free diet to small holders.

From the 7th day of age changes were made in the diet of the different groups, as it is shown below in Table 5:

Table 5. The different treatments used in broiler experiment with medicinal herb mixture pre-treatment

Groups	Diet	No. of birds
Control (C)	Intensive grower diet (from 7th day of age)	21x2
Control (C)*, ***** later: Treated I.	Intensive grower diet (from 7th until 21st day of age)	21x2
Treated II. (**With low dose of Herbamix treated [H1]**)	Intensive grower diet + Herbamix Basic Premix™ (300 mg/kg) from 7th day of age	21x2
Treated III. (**With medium dose of Herbamix treated [H2]**)	Intensive grower diet + Herbamix Basic Premix™ (600 mg/kg) from 7th day of age	21x2
Treated IV. (**With high dose of Herbamix treated [H5]**)	Intensive grower diet + Herbamix Basic Premix™ (1500 mg/kg) from 7th day of age	21x2

From the 21st day of age the treated groups received aflatoxin contaminated feed as it is shown below:

Groups	Diet	No. of birds
Control (C)	Intensive grower diet	12x2
Treated I. **(Aflatoxin treated [A])**	Intensive grower diet + aflatoxin (140 µg AFB_1/kg)	12x2
Treated II. (**With low dose of Herbamix + Aflatoxin treated [H1+A]**)	Intensive grower diet + Herbamix Basic Premix™ (300 mg/kg) + aflatoxin (140 µg AFB_1/kg)	12x2
Treated III. (**With medium dose of Herbamix + Aflatoxin treated [H2+A]**)	Intensive grower diet + Herbamix Basic Premix™ (600 mg/kg) + aflatoxin (140 µg AFB_1/kg)	12x2
Treated IV. (**With high dose of Herbamix + Aflatoxin treated [H5+A]**)	Intensive grower diet + Herbamix Basic Premix™ (1500 mg/kg) + aflatoxin (140 µg AFB_1/kg)	12x2

The light program followed the guidelines for Cobb 540 broilers until the start of the experiment, then continuous light regimen was used for the whole period (48 hours) of mycotoxin exposure.

4.2.2.2. Preparation of feed

4.2.2.2.1. Preparation of herbal mixture containing feed

Herbal mixture (Herbamix Basic Premix™, Herbamix Trade Ltd., Budapest) was added to the complete feed in powder form at 3 different doses as formerly described in Chapter 4.2.1.1.1.

4.2.2.2.2. Artificial mycotoxin contamination of the feed

Aflatoxin was produced by *Aspergillus flavus* strain (ZT80) isolated by Dobolyi et al. (2013), on ground corn substrate. The corn substrate, with known aflatoxin concentration was mixed to the feed in order to reach the final concentration of intended doses. Aflatoxin content of the loaded complete feeds was analyzed with AFLAPREP HPLC method after immunoaffinity clean-up (Food Analytica Ltd., Gyula).

Measured total aflatoxin concentration of the commercial diet was <1.0 µg/kg. Measured total aflatoxin concentration of the aflatoxin contaminated feeds were: 149.7 µg/kg; in which AFB_1: 140 µg/kg; AFB_2: 8.6 µg/kg; AFG_1: <1.0 µg/kg; AFG_2: 1.1 µg/kg.

4.2.2.3. Sampling and weighing

The short-term trial lasted for 2 days, after 12 hours of feed deprivation. Six randomly chosen birds were exterminated from each group at 12^{th}, 24^{th}, 36^{th} and 48^{th} hours of the experiment.

The sampling and weighing protocol was identical to the one shown earlier in chapter 4.2.1.3.

4.3. Biochemical methods

4.3.1. Measurement of reduced glutathione concentration

Reduced glutathione (GSH) concentration of blood plasma, red blood cell haemolysate, and 10,000 g supernatant fraction of liver and kidney homogenate samples was determined after deproteinization with 10% w/v trichloro-acetic acid (TCA) (Carlo Erba, Rodano). The samples were centrifuged (10,000 g, 3 min), and the supernatant fraction was used for color complex formation of glutathione with Ellmann's reagent (5,5'-dithiobis-2 nitrobensoic acid, DTNB) (Sigma, St. Louis) using the method of Sedlak and Lindsay (1968). The concentration of non-protein sulfhydryl group (-SH) is spectrophotometrically measurable with the measurement of the yellowish color complex at 412 nm. The optimal pH (pH=8.0-8.2) for the complex formation was set by tris(hydroxymethyl) aminomethane (TRIS) buffer (Sigma, St. Louis). The GSH concentration was calculated to protein content of the samples investigated.

4.3.2. Measurement of glutathione peroxidase activity

Glutathione peroxidase (GPx) (E.C.1.11.1.9) activity of blood plasma, red blood cell haemolysate, and 10,000 g supernatant fraction of liver and kidney homogenate samples was measured using reduced glutathione (Sigma, St. Louis) and cumene-hydroperoxide (Merck, Darmstadt) as co-substrates in an end point direct assay as described by Matkovics et al. (1988). The length of this enzymatic reaction was 10 minutes, which was followed by deproteinization with 10% w/v trichloro-acetic acid (TCA) (Carlo Erba, Rodano). The loss of GSH was measured with Ellmann's reagent, measuring the light absorbance of the formed complex at 412 nm. The enzyme activity was expressed as units (U), which means nmol glutathione oxidation per minute at 25 ^{o}C. The enzyme activity was calculated to protein content of the samples investigated.

4.3.3. Measurement of total protein concentration

In case of blood plasma and red blood cell haemolysate samples protein concentration was determined by the biuret method, using the reagent kit (No. 41951) of Diagnosticum Ltd. (Budapest, Hungary). In case of the biuret method (Weichselbaum, 1948) the copper(II) ions react with the peptide bonds of proteins in alkali condition, generating a colored complex. The light absorbance of the blue color complex was measured at 546 nm. DunaCal human multicalibrator standard (Diagnosticum Ltd., Budapest) was used as standard.

For the determination of protein concentration in 10,000 g supernatant fraction of tissue (liver and kidney) homogenates, the method of Lowry et al. (1951) was used.

This method is based on two reactions. At first step, the copper(II) ions are reduced to copper(I) ions. At second phase the tyrosine and tryptophan residues reduce the Folin-Ciocalteu reagents (phosphorus molibdate and phosphorus wolframate), to form well detectable blue color, which's absorbance is measurable at 750 nm. Bovine serum albumin (Sigma, St. Louis) was used as standard.

4.3.4. Measurement of conjugated diene (CD) and –triene (CT) content

Level of conjugated dienes (CD) and conjugated trienes (CT) as markers of initial phase of lipid peroxidation, was measured in liver, according to the method of AOAC (1984). Small amount (0.1 g) of liver was homogenized in 2,2,4-trimethylpentane (Sigma, St. Louis) and left overnight in dark place. The absorbance of samples was measured at 232 nm (for CD) and 268nm (for CT) after extraction. The amount of CD and CT expressed as OD, due to lack of appropriate standard.

4.3.5. Measurement of malondialdehyde (MDA) concentration

Malondialdehyde (MDA) concentration of blood plasma and red blood cell haemolysate samples was measured based on the color complex formation of malondialdehyde with 2- thiobarbituric acid in an acidic environment at high temperature (Placer et al., 1966).

Malondialdehyde concentrations in the native tissue (liver, kidney) homogenates were determined with colorimetric method according to Mihara et al. (1980) as modified by Matkovics et al. (1988). The standard for these measurements was 1,1,3,3-tetraethoxypropane (Fluka, Buchs).

4.3.6. Determination of aspartate aminotransferase (AST, GOT) activity

For measurement of the aspartate aminotransferase (AST, GOT) activity of blood plasma samples a reagent kit (No. 46263) of Diagnosticum Ltd. (Budapest, Hungary) was used. The protocol is based on the method of IFCC (1976). Two substrates participate in the reaction catalyzed by AST, namely L-aspartate and alpha-ketoglutarate, forming L-glutamate and oxalacetate. The formed oxalacetate is converted to L-malate and NAD+ by malate dehydrogenase (MDH) in prescence of NADH coenzyme. The oxido-reductive conversion of NADH/NAD+ is indicated by a decrease in absorbance at 340 nm in this kinetic method.

4.3.7. Determination of gamma-glutamyltransferase (GGT) activity

To determine the gamma-glutamyltransferase (GGT) activity of blood plasma samples a reagent kit (No. 47263) of Diagnosticum Ltd. (Budapest, Hungary) was used. The protocol is based on the method of Szasz (1976). Gamma-glutamyltransferase catalyzes the transfer of the γ-glutamyl group from L-γ-glutamil-3-carboxy-4-nitroanilide substrate to glycylglycine. The amount of released p-nitroaniline is proportional to the γ-GT activity of serum, which can be measured spectrophotometrically at 405 nm in this kinetic method.

4.3.8. Determination of glucose concentration

For measurement of the glucose concentration of blood plasma samples a commercial reagent kit (No. 46861) of Diagnosticum Ltd. (Budapest, Hungary) was used. The process is based on the method of Trinder (1969), with modification of the original method by Barham and Trinder (1972). In presence of water and oxygen, glucose is converted into gluconate and hydrogen-peroxide by glucose oxidase

(GOD). In presence of phenol and 4-aminoantipyrine, the produced hydrogen-peroxide (H_2O_2) is degraded by peroxidase (POD) to a red color product, which is spectrophotometrically measurable at 505 nm. The increase in absorbance correlates with the glucose concentration of the blood plasma sample.

4.3.9. Determination of cholesterol concentration

To determine the cholesterol concentration of blood plasma samples a commercial reagent kit (No. 41031) of Diagnosticum Ltd. (Budapest, Hungary) was used. The protocol of this analysis is based on the method of Allain et al. (1974). The cholesterol esters of the blood plasma sample are hydrolyzed by cholesterol esterhydrolase (ChEH) to form cholesterol and fatty acids. In presence of oxygen cholesterol is converted to 4-cholesten-3-one and hydrogen-peroxide (H_2O_2) by cholesterol oxidase (ChOD). As it is described earlier, in case of the glucose measurement, the produced hydrogen-peroxide (H_2O_2) is degraded by peroxidase (POD) in presence of phenol and 4-aminoantipyrine to a red quinonimine derivative which is spectrophotometrically measurable at 505 nm. The increase in absorbance correlates with the cholesterol concentration of the blood plasma sample.

4.3.10. Determination of triglyceride concentration

For measurement of concentration of triglycerides in blood plasma samples a reagent kit (No. 47163) of Diagnosticum Ltd. (Budapest, Hungary) was used. The process is based on the method of Young et al. (1975) modified by Fossati and Prencipe (1982). The triglycerides in the blood plasma sample are hydrolyzed to glycerol and fatty acids by lipoprotein lipase (LPL). The produced glycerol is then phosphorylated by glycerol kinase (GK) in the presence of ATP and Mg^{2+} ions. The formed glycerol-3-phosphate is oxidized by glycerol-3-phosphate oxidase (GPO) in the presence of molecular oxygen (O_2). During the next reaction catalysed by peroxidase (POD), a red quinonimine derivate is formed from hydrogen-peroxide (H_2O_2) in presence of 4-aminoantipyrine and p-chlorophenol, which is spectrophotometrically measurable at 505 nm. The increase in absorbance correlates with the triglyceride concentration of the blood plasma sample.

4.3.11. Determination of uric acid concentration

For measurement of the uric acid concentration of blood plasma samples a reagent kit (No. 46763) of Diagnosticum Ltd. (Budapest, Hungary) was used. The process is based on the method of Trivedi et al. (1978). In the first reaction the uric acid is converted to allantoin by uricase, while carbon dioxyde and hydrogen-peroxide (H_2O_2) are formed. During the next reaction a purple colored oxidized condensation product is formed from hydrogen-peroxide in presence of 4-aminoantipiryne and N-Ethyl-N-(3-sulfopropyl)-3-methoxyaniline (ADPS), which is spectrophotometrically measurable at 546 nm. The increase in absorbance correlates with the uric acid concentration of the blood plasma sample.

4.4. Ethical issues

The animal experiments were carried out according to the Hungarian Animal Protection Act, in compliance with the EU rules, except the continous light regimen in short term trials with broiler chicken. The experimental protocols were authorized by the Food Chain Safety and Animal Health Directorate of the Pest County Agricultural Office, under permission number XIV-1-001/1880-5/2011.

4.5. Statistical analysis

For statistical analysis of data, the GraphPad Prism 5.04 for Windows (GraphPad Software, San Diego, CA, USA) was used. After calculating the mean and standard deviation (SD) values, all data were subjected to one-way variance analysis (ANOVA), and differences between means was calculated with the Tukey test at a 95% interval of confidence ($p<0.05$). In tables and diagrams data are reported as mean±SD. If significant differences were detected between the groups, the lowest mean values were signed with ‘a’ letter in superscript. Different letters (e.g. ‘a’, ‘b’, ‘c’) in superscript represent significant difference at $p<0.05$.

5. RESULTS AND DISCUSSION

5.1. Effects of a medicinal herb mixture on short-term effects of T-2 toxin or DON on some lipid peroxide and glutathione redox status parameters in broiler chicken

5.1.1. Results

Consumption of high T-2 toxin/HT-2 toxin contaminated diet (20 times higher than the EU recommendation) or the high DON exposure (3.22 times higher than the EU recommendation) did not cause mortality during the short-term trial, and no clinical signs of toxicity were observed.

Calculated feed intake showed some differences at different periods of the trial, but feed intake was measured for groups, therefore no statistical analysis could be done on this parameter (Table 6).

Calculated mycotoxin intake, which was calculated from feed intake and measured mycotoxin content of the particular complete feed, was almost the same between the groups fed with mycotoxin contaminated diets (Table 7 and Table 8).

The measured transit time of the methyl-orange containing control feed in this experiment was 5 hours 40 minutes.

Table 6. Individual and combined effect of T-2 toxin or DON and medicinal herb mixture (Herbamix™) on calculated feed intake of broiler chickens

Experimental group	Calculated feed intake per broiler chicken (g/bird)				
	0-12 h	**12-24 h**	**24-36 h**	**36-48 h**	**0-48h**
Control	68.13	42.78	75.00	87.50	273.41
T-2 toxin	65.42	46.39	47.92	104.17	263.90
DON	70.63	50.28	59.58	134.17	314.66
Herbamix	66.67	51.94	52.50	123.33	294.44
T-2 toxin + Herbamix	68.54	47.50	47.50	115.00	278.54
DON + Herbamix	68.33	51.67	56.25	120.00	296.25

T-2 toxin: 3.72 mg T-2 toxin/kg feed and 1.26 mg HT-2 toxin/kg feed; DON: 16.12 mg DON/kg feed; Herbamix: 600 mg Herbamix Basic Premix/kg feed

Table 7. Calculated mycotoxin intake of broiler chickens

Group	Calculated mycotoxin intake (mg/bird)									
	0-12 h		**12-24 h**		**24-36 h**		**36-48 h**		**0-48 h**	
	DON	T-2 *HT-2*	DON	T-2 *HT-2*	DON	T-2 *HT-2*	DON	T-2 *HT-2*	DON	T-2 *HT-2*
Control	0.017		0.011		0.019		0.022		0.068	
T-2 toxin		0.245 *0.082*		0.174 *0.059*		0.179 *0.060*		0.390 *0.131*		0.987 *0.336*
DON	1.139		0.811		0.960		2.163		5.072	
Herbamix	0.017		0.013		0.013		0.031		0.074	
T-2 toxin + Herbamix		0.256 *0.086*		0.178 *0.060*		0.178 *0.060*		0.430 *0.145*		1.042 *0.351*
DON + Herbamix	1.102		0.833		0.907		1.934		4.776	

T-2 toxin: 3.72 mg T-2 toxin/kg feed and 1.26 mg HT-2 toxin/kg feed; DON: 16.12 mg DON/kg feed; Herbamix: 600 mg Herbamix Basic Premix/kg feed

Table 8. Calculated mycotoxin intake of broiler chickens per kg body weight

Group	Calculated mycotoxin intake (µg/kg BW)									
	0-12 h		**12-24 h**		**24-36 h**		**36-48 h**		**0-48 h**	
	DON	T-2 *HT-2*	DON	T-2 *HT-2*	DON	T-2 *HT-2*	DON	T-2 *HT-2*	DON	T-2 *HT-2*
Control	22.0		14.8		24.3		28.2		89.4	
T-2 toxin		334.8 *112.8*		209.0 *70.4*		240.0 *80.9*		528.3 *178.0*		1312.1 *442.0*
DON	1483.5		1137.6		1204.3		2552.0		6377.3	
Herbamix	21.9		17.7		16.9		36.4		92.9	
T-2 toxin + Herbamix		370.6 *124.8*		221.4 *74.6*		231.5 *78.0*		570.9 *192.3*		1394.4 *469.8*
DON + Herbamix	1427.3		1110.6		1221.2		2366.2		6125.4	

T-2 toxin: 3.72 mg T-2 toxin/kg feed and 1.26 mg HT-2 toxin/kg feed; DON: 16.12 mg DON/kg feed; Herbamix: 600 mg Herbamix Basic Premix/kg feed

Neither body weights nor absolute and relative liver weights showed significant differences during the trial (Table 9).

Table 9. Individual and combined effect of T-2 toxin, DON and herbal product on body weight, liver weight and relative liver weight of broiler chickens (mean±SD; n=6)

	Control	**T-2 toxin**	**DON**	**Herbamix**	**T-2 toxin + Herbamix**	**DON+ Herbamix**
			body weight (g)			
12th hour	774.20± 65.68	730.80± 93.56	767.50± 112.60	761.70± 145.90	691.70± 55.92	771.70± 72.23
24th hour	723.30± 98.83	830.00± 167.90	712.50± 171.70	731.70± 80.66	802.50± 85.54	750.00± 128.60
36th hour	770.00± 100.30	746.70± 102.60	797.50± 93.95	778.30± 134.00	767.50± 94.91	742.50± 187.50
48th hour	775.00± 52.15	737.50± 172.40	847.50± 116.40	845.80± 84.46	753.30± 58.88	817.50± 99.34
			liver weight (g)			
12th hour	23.55± 3.98	23.62± 4.90	26.00± 4.81	24.05± 5.29	20.59± 2.94	27.32± 4.70
24th hour	19.25± 3.61	26.63± 5.82	20.79± 6.66	20.52± 2.84	24.71± 4.68	23.89± 6.80
36th hour	21.90± 3.80	18.64± 3.46	21.21± 2.58	18.93± 3.71	19.86± 4.60	21.77± 7.46
48th hour	17.58± 2.21	16.61± 4.52	21.45± 2.80	19.17± 2.30	17.52± 1.62	19.01± 2.77
			relative liver weight (g/100 g body weight)			
12th hour	3.03± 0.32	3.22± 0.52	3.39± 0.43	3.16± 0.25	2.98± 0.31	3.54± 0.48
24th hour	2.65± 0.26	3.21± 0.16	2.91± 0.44	2.81± 0.26	3.06± 0.35	3.16± 0.56
36th hour	2.84± 0.31	2.50± 0.33	2.66± 0.18	2.43± 0.16	2.58± 0.46	2.89± 0.32
48th hour	2.28± 0.33	2.24± 0.14	2.54± 0.17	2.27± 0.18	2.32± 0.08	2.32± 0.18

T-2 toxin: 3.72 mg T-2 toxin/kg feed and 1.26 mg HT-2 toxin/kg feed; DON: 16.12 mg DON/kg feed; Herbamix: 600 mg Herbamix Basic Premix/kg feed

5.1.1.1. Parameters of glutathione redox system and lipid peroxidation processes in blood plasma and RBC haemolysate

Concentration of malondialdehyde (MDA), one of the end products of lipid peroxidation, did not change significantly in blood plasma and in RBC haemolysate (Table 10 and Table 11). No significant changes were found in reduced glutathione (GSH) concentration of blood plasma and RBC haemolysate, as well (Table 10 and Table 11).

Glutathione peroxidase (GPx) activity of blood plasma showed marked changes at 12 and 48 hours of mycotoxin exposure (Table 10). At hour 12 it was lower in DON + herbal mixture treated group as compared to T-2 toxin + herbal mixture group, and at hour 48 herbal mixture alone caused significantly lower enzyme activity than the control (Table 10).

Table 10. Individual and combined effect of T-2 or DON toxins and herbal mixture on lipid peroxidation and glutathione redox system of blood plasma (mean±SD; n=6)

	Control	**T-2 toxin**	**DON**	**Herbamix**	**T-2 toxin + Herbamix**	**DON + Herbamix**
			MDA (µmol/L)			
12th hour	21.02± 5.47	18.84± 1.24	19.06± 2.14	17.09± 2.77	18.01± 1.87	17.44± 1.03
24th hour	18.88± 5.30	15.45± 1.18	16.71± 2.19	17.32± 1.33	17.03± 1.27	16.17± 1.11
36th hour	17.72± 5.24	16.41± 7.06	19.53± 5.36	16.32± 5.41	15.39± 5.09	17.53± 5.21
48th hour	19.06± 1.48	18.49± 1.26	19.71± 2.34	18.58± 1.40	20.16± 3.17	21.16± 1.30
			GSH (µmol/g protein content)			
12th hour	9.40± 1.58	9.84± 1.59	8.44± 1.69	9.28± 1.22	9.40± 2.21	7.66± 2.03
24th hour	5.73± 1.04	6.97± 1.25	5.23± 2.38	7.39± 1.45	6.53± 0.45	6.30± 0.43
36th hour	5.24± 0.84	7.06± 2.08	5.36± 1.33	5.41± 0.94	5.09± 0.44	5.21± 1.00
48th hour	5.45± 0.79	5.12± 1.06	4.82± 0.49	4.70± 0.30	5.31± 0.41	4.96± 0.48
			GPx (U/g protein content)			
12th hour	10.0^{ab}± 1.42	9.88^{ab}± 0.74	8.16^{ab}± 1.94	8.45^{ab}± 1.13	10.27^{b}± 1.88	8.06^{a}± 1.85
24th hour	9.80± 0.83	11.45± 2.72	9.52± 2.94	11.92± 2.74	10.53± 0.55	10.82± 0.64
36th hour	7.41± 1.47	9.61± 2.60	6.96± 1.33	7.56± 2.06	8.53± 1.60	8.39± 1.83
48th hour	11.00^{b}± 2.86	10.37^{ab}± 2.81	8.35^{ab}± 1.64	7.88^{a}± 0.98	8.38^{ab}± 0.56	8.30^{ab}± 1.40

T-2 toxin: 3.72 mg T-2 toxin/kg feed and 1.26 mg HT-2 toxin/kg feed; DON: 16.12 mg DON/kg feed; Herbamix: 600 mg Herbamix Basic Premix/kg feed

^{a,b} Means designated with different letters within the same row mean significant difference ($p<0.05$)

Table 11. Individual and combined effect of T-2 or DON toxins and herbal mixture on lipid peroxidation and glutathione redox system of red blood cell haemolysates (mean±SD; n=6)

	Control	**T-2 toxin**	**DON**	**Herbamix**	**T-2 toxin + Herbamix**	**DON + Herbamix**
			MDA (µmol/L)			
12th hour	41.51± 9.42	36.22± 6.41	35.81± 9.98	33.40± 11.60	36.68± 12.17	33.69± 10.14
24th hour	27.02± 4.28	30.08± 4.86	32.06± 3.89	27.22± 3.58	27.34± 5.21	25.80± 2.35
36th hour	30.31± 8.04	29.54± 4.98	32.86± 8.54	35.18± 2.76	35.24± 1.87	33.67± 4.36
48th hour	37.51± 4.96	38.21± 8.80	39.50± 12.94	33.76± 4.42	36.78± 8.56	36.52± 7.69
			GSH (µmol/g protein content)			
12th hour	7.46 2.31	10.34± 3.25	9.24± 1.63	9.30± 3.25	9.07± 1.04	8.61± 0.71
24th hour	7.91± 1.40	9.05± 1.85	8.38± 1.93	8.51± 1.10	8.44± 1.83	8.90± 1.67
36th hour	7.18± 1.86	7.74± 3.27	6.58± 1.06	7.14± 3.64	6.74± 1.67	7.37± 1.41
48th hour	7.76± 1.47	8.65± 2.23	7.72± 1.18	9.62± 2.32	8.62± 2.00	7.73± 1.46
			GPx (U/g protein content)			
12th hour	4.76± 0.78	4.94± 0.69	4.78± 0.41	4.94± 0.69	5.30± 0.63	5.09± 0.58
24th hour	6.62± 1.07	6.77± 1.38	6.90± 0.86	6.53± 0.42	6.72± 1.80	6.92± 0.98
36th hour	5.82± 1.37	5.21± 1.03	4.19± 0.49	4.94± 1.84	5.57± 2.07	6.65± 1.43
48th hour	6.97± 2.49	6.15± 1.54	5.02± 1.18	6.75± 1.01	6.55± 0.94	6.12± 0.96

T-2 toxin: 3.72 mg T-2 toxin/kg feed and 1.26 mg HT-2 toxin/kg feed; DON: 16.12 mg DON/kg feed; Herbamix: 600 mg Herbamix Basic Premix/kg feed

[a,b] Means designated with different letters within the same row mean significant difference ($p<0.05$)

As effect of T-2 toxin/HT-2 toxin or DON, level of conjugated dienes and conjugated trienes, the initial phase markers of lipid peroxidation processes, did not change significantly in liver, and it was not modified by the supplementation of herbal mixture (Table 12).

Table 12. Individual and combined effect of T-2 or DON toxins and herbal mixture on conjugated diene (CD) and conjugated triene (CT) levels in liver (mean±SD; n=6)

	Control	**T-2 toxin**	**DON**	**Herbamix**	**T-2 toxin + Herbamix**	**DON + Herbamix**
				CD (OD 232nm)		
12th hour	0.28± 0.02	0.26± 0.02	0.28± 0.01	0.27± 0.01	0.28± 0.02	0.26± 0.01
24th hour	0.34± 0.11	0.36± 0.12	0.28± 0.02	0.39± 0.11	0.33± 0.12	0.37± 0.15
36th hour	0.24± 0.01	0.23± 0.01	0.23± 0.02	0.24± 0.01	0.23± 0.01	0.22± 0.02
48th hour	0.25± 0.02	0.24± 0.02	0.25± 0.02	0.24± 0.01	0.23± 0.03	0.23± 0.03
				CT (OD 268nm)		
12th hour	0.15± 0.01	0.14± 0.02	0.15± 0.01	0.14± 0.01	0.15± 0.01	0.14± 0.01
24th hour	0.17± 0.02	0.18± 0.02	0.15± 0.01	0.18± 0.02	0.17± 0.03	0.16± 0.04
36th hour	0.12± 0.01	0.12± 0.01	0.12± 0.01	0.12± 0.01	0.12± 0.01	0.11± 0.01
48th hour	0.13± 0.01	0.12± 0.01	0.13± 0.01	0.12± 0.01	0.12± 0.02	0.11± 0.02

T-2 toxin: 3.72 mg T-2 toxin/kg feed and 1.26 mg HT-2 toxin/kg feed; DON: 16.12 mg DON/kg feed; Herbamix: 600 mg Herbamix Basic Premix/kg feed

5.1.1.2. Parameters of glutathione redox system and lipid peroxidation processes of tissue homogenates

Concentration of MDA, the meta-stable end product of lipid peroxidation, was significantly lower in the liver homogenate of both mycotoxin loaded groups fed with herbal mixture supplemented feed at 24th hour sampling as compared to the control (Table 13).

GSH concentration in liver homogenate showed differences at 24-hour sampling, out of which T-2 toxin caused significant difference, and T-2 toxin + herbal mixture combination caused markedly, but not significantly, higher values than the control. On the other hand, in the case of DON significantly higher GSH content was measured only when it was combined with herbal mixture supplementation (Table 13).

In liver homogenate GPx activity changed significantly at 24-hour sampling, when higher values were found as effect of T-2 toxin, also in combination with herbal mixture, and DON in combination with herbal mixture, as compared to the control, and in case of DON, when it was used alone (Table 13).

Table 13. Individual and combined effect of T-2 or DON toxins and herbal mixture on lipid peroxidation and glutathione redox system of liver homogenates (mean±SD; n=6)

	Control	**T-2 toxin**	**DON**	**Herbamix**	**T-2 toxin + Herbamix**	**DON + Herbamix**
			MDA (nmol/g fresh weight)			
12th hour	10.99± 2.15	10.12± 3.02	9.36± 0.54	9.74± 2.10	9.15± 2.40	9.88± 4.47
24th hour	17.95^{b}± 3.69	12.62^{ab}± 1.88	15.00^{ab}± 4.94	14.42^{ab}± 3.51	11.99^{a}± 2.60	11.72^{a}± 1.61
36th hour	10.05± 1.37	11.37± 2.31	10.04± 2.12	10.15± 2.33	10.69± 0.90	9.16± 2.49
48th hour	12.57± 3.47	15.51± 2.68	12.03± 3.24	11.81± 3.64	12.46± 2.31	14.54± 6.23
			GSH (µmol/g protein content of 10,000 g supernatant)			
12th hour	3.03± 0.75	4.03± 1.12	3.55± 0.89	3.15± 0.27	3.22± 0.51	3.19± 0.77
24th hour	2.84^{a}± 1.07	4.56^{bc}± 0.74	3.00^{ab}± 1.17	3.55^{abc}± 0.70	4.17^{abc}± 0.86	4.51^{b}± 0.50
36th hour	3.46± 0.83	3.60± 0.58	3.08± 0.60	2.93± 0.39	3.42± 0.32	3.19± 0.48
48th hour	2.62± 0.40	2.92± 0.51	3.15± 0.58	3.03± 0.66	2.82± 0.30	2.74± 0.29
			GPx (U/g protein content of 10,000 g supernatant)			
12th hour	3.01± 0.66	3.99± 0.91	3.48± 0.68	3.14± 0.21	3.21± 0.53	3.07± 1.35
24th hour	3.10^{a}± 1.24	4.78^{b}± 0.67	2.89^{a}± 0.96	3.51^{ab}± 0.94	4.69^{b}± 0.74	4.70^{b}± 0.44
36th hour	3.23± 0.88	3.44± 0.61	3.16± 0.73	3.06± 0.63	3.40± 0.44	3.23± 0.62
48th hour	1.99± 0.37	2.54± 0.48	2.84± 0.49	2.64± 0.58	2.72± 0.61	2.31± 0.37

T-2 toxin: 3.72 mg T-2 toxin/kg feed and 1.26 mg HT-2 toxin/kg feed; DON: 16.12 mg DON/kg feed; Herbamix: 600 mg Herbamix Basic Premix/kg feed

^{a,b,c} Means designated with different letters within the same row mean significant difference ($p<0.05$)

In kidney homogenates significant differences were found in MDA concentration at 12 hour sampling, when DON alone and in combination with herbal mixture revealed the highest values which were significantly higher than in T-2 toxin + herbal mixture treated group (Table 14).

GSH concentration of kidney homogenates showed significant difference at 48 hour sampling, when significantly higher value was revealed in T-2 toxin + herbal mixture group as compared to the control (Table 14).

GPx activity in kidney homogenates showed significant differences at 48 hour sampling, where T-2 toxin load resulted in higher activity when it was used together with herbal mixture as compared to herbal mixture supplemented group (Table 14).

Table 14. Individual and combined effect of T-2 or DON toxins and herbal mixture on lipid peroxidation and glutathione redox system of kidney homogenates (mean±SD; n=6)

	Control	**T-2 toxin**	**DON**	**Herbamix**	**T-2 toxin + Herbamix**	**DON + Herbamix**
			MDA (nmol/g fresh weight)			
12th hour	8.02^{ab}± 1.77	9.80^{ab}± 1.62	10.34^{b}± 2.33	8.30^{ab}± 3.08	6.25^{a}± 0.43	10.30^{b}± 1.77
24th hour	11.21± 1.90	9.76± 0.62	9.30± 1.23	9.91± 2.13	9.40± 1.07	8.85± 1.35
36th hour	9.40± 1.49	10.10± 1.03	9.66± 2.54	9.40± 1.13	7.82± 2.34	9.68± 1.79
48th hour	12.24± 1.15	11.21± 1.36	10.51± 2.59	10.76± 1.12	9.63± 0.43	10.36± 1.13
			GSH (µmol/g protein content of 10,000 g supernatant)			
12th hour	5.51± 0.35	6.09± 0.67	5.91± 0.96	5.81± 1.15	5.66± 0.26	5.76± 0.77
24th hour	4.74± 0.50	4.94± 0.97	4.50± 0.49	4.36± 0.52	4.95± 0.39	4.75± 0.50
36th hour	3.56± 0.43	3.30± 1.69	4.19± 0.52	3.49± 0.37	3.92± 1.24	3.63± 0.94
48th hour	4.38^{a}± 0.70	5.14^{ab}± 0.55	5.04^{ab}± 0.81	4.65^{ab}± 0.63	5.77^{b}± 1.05	5.56^{ab}± 0.78
			GPx (U/g protein content of 10,000 g supernatant)			
12th hour	4.50± 1.01	4.90± 0.87	5.00± 0.79	4.71± 0.71	4.99± 0.30	4.85± 0.61
24th hour	4.72± 0.49	4.72± 0.87	4.45± 0.65	4.08± 0.73	4.82± 0.32	4.66± 0.38
36th hour	4.91± 0.83	4.73± 1.90	5.24± 0.22	4.49± 0.66	5.16± 1.32	4.86± 1.15
48th hour	4.02^{ab}± 0.52	4.14^{ab}± 0.36	3.89^{a}± 0.63	3.89^{a}± 0.64	4.98^{b}± 0.67	4.65^{ab}± 0.57

T-2 toxin: 3.72 mg T-2 toxin/kg feed and 1.26 mg HT-2 toxin/kg feed; DON: 16.12 mg DON/kg feed; Herbamix: 600 mg Herbamix Basic Premix/kg feed

^{a,b} Means designated with different letters within the same row mean significant difference ($p<0.05$)

5.1.2. Discussion

No clinical signs of toxicity and mortality were observed at the dose of trichothecenes applied, which is supported by the relatively high tolerance of broiler chicken to DON (Dänicke et al., 2001) or T-2 toxin (Eriksen and Pettersson, 2004). There were some differences in the calculated feed intake at different periods of the trial, which probably are caused by the well-known feed refusal effect of T-2 toxin (Hoerr, 2008). Absolute or relative liver weight also did not change as effect of mycotoxin exposure, which is supported by a previous study where 1 or 5 mg DON/kg feed showed the same results (Awad et al., 2011a).

Early markers of lipid peroxidation, levels of conjugated dienes and trienes did not differ significantly among the experimental groups as effect of mycotoxin exposure in liver, and herbal mixture also had no effect on these parameters. However, end-product of the process, the MDA content was markedly lower in the liver as effect of DON or T-2 toxin, which is contrary to the results of some previous long-term studies at low doses (Awad et al., 2014; Mézes et al., 1998; Osselaere et al., 2013), but supported by some others (Frankic et al., 2006; Rezar et al., 2007) using high doses of DON or T-2 toxin. The results revealed that addition of herbal mixture reduced the MDA values, which is probably due to the lack of marked oxidative stress in the liver as effect of the mycotoxin at doses used in this trial. In kidney, at 12^{th} hour of mycotoxin exposure MDA concentration was the highest in DON treated group, followed by the T-2 toxin treated group. Herbal mixture had no effect in combination with DON, but it has reduced the MDA concentration together with T-2 toxin exposure, which suggests that antioxidant effect of herbal mixture is adequate only at low level of free radical formation as caused by trichothecene mycotoxins.

GSH content in liver homogenate showed higher values when T-2 toxin contaminated feed was fed, alone or in combination with herbal mixture. It means that the moderate oxidative stress in the liver, as effect of T-2 toxin, activates the glutathione synthesis, as part of the antioxidant response (Zimniak et al., 1997). This result is important in aspect that herbal mixture induces the synthesis of GSH in the liver of broiler chicken, which is the main site of GSH biosynthesis (Shelly and Lu, 2013). In the case of DON exposure, higher GSH level was found only in combination with herbal mixture, which might refer to the potential antioxidant effect of active constituents of the herbal mixture.

In kidney, DON had no measurable effect on GSH content, possibly because of the higher oxidative stress, but T-2 toxin, in combination with herbal mixture, resulted in increased GSH level at the end of the trial, which suggests that longer period is required for the adaptation to mild oxidative stress, and this supports the antioxidant effect of herbal mixture. Actual GSH concentration also depends on the reduction of its oxidised form, glutathione disulphide by glutathione reductase enzyme, but this parameter was not measured in this trial.

Glutathione peroxidase activity in liver homogenate changed significantly at 24-hour sampling, when higher values were found as effect of T-2 toxin, also in combination with herbal mixture and of DON in combination with herbal mixture. It means that T-2 toxin activates the enzymatic antioxidant defence, in this case GPx activity, but herbal mixture had no additional effect, but when it was used alone, such effect was not manifested. In kidney homogenate significant changes were found at 48-hour sampling, as T-2 toxin load resulted in higher activity when it was used together with herbal

mixture, as compared to herbal mixture supplemented group. These results agree with the ones found in liver, where T-2 toxin induced the activity of glutathione peroxidase, without additional effect of herbal mixture. Lack of effect of DON probably can be explained with the high tolerance of broiler chicken to DON, which is probably caused by the higher rate of deepoxidation in the gastrointestinal tract or in the liver (Awad et al., 2014).

In conclusion, the results the investigated ’type A’ trichothechene mycotoxin, T-2 toxin induced oxygen free radical formation with low or moderately high efficiency, and consequently activated the glutathione redox system in liver and kidney of broiler chicken. Addition of herbal mixture has only moderate effect against the mild oxidative stress caused by DON or T-2 toxin at the dose applied.

5.2. Effect of short-term aflatoxin exposure in combination with medicinal herb mixture (Herbamix™) on lipid peroxidation and glutathione redox system in laying hens

5.2.1. Results

Consumption of high aflatoxin contaminated diet (where the AFB_1 concentration was 6.25 times higher than the EU regulation) did not cause morbidity or mortality in the experimental groups during this short-term study, and no clinical signs of aflatoxicosis were observed.

No marked change was observed in calculated feed intake of the layers (Table 15), therefore the calculated total aflatoxin intake, which was calculated from feed intake and measured mycotoxin content of the complete feed, was almost the same between the groups fed with aflatoxin contaminated diet and aflatoxin contaminated and herbal mixture supplemented diet (Table 16 and Table 17). Due to the darkness there was no measurable feed intake in the periods of 12^{th} to 24^{th} hour, and 36^{th} to 48^{th} hour (Table 15), therefore aflatoxin intake was also negligible during these periods (Table 16 and Table 17).

The measured transit time of the methyl-orange containing control feed in this experiment was 4 hours 10 minutes.

Table 15. Individual and combined effect of aflatoxin B_1 and medicinal herb mixture (Herbamix™) on calculated feed intake of laying hens

Experimental group	Calculated feed intake per laying hen (g/bird)				
	0-12 h	**12-24 h**	**24-36 h**	**36-48 h**	**0-48 h**
Control	146.50	3.06	144.50	5.50	299.56
Herbamix	150.00	0.00	147.50	2.50	300.00
Aflatoxin	150.00	0.00	146.50	0.83	297.33
Aflatoxin + Herbamix	146.25	1.56	140.50	8.33	296.65

Aflatoxin: 125 µg AFB_1/kg feed; Herbamix: 600 mg Herbamix Basic Premix/kg feed

Table 16. Calculated mycotoxin intake of laying hens

Group	Calculated total aflatoxin intake (µg/bird)				
	0-12 h	**12-24 h**	**24-36 h**	**36-48 h**	**0-48 h**
Control	0.15	0.00	0.14	0.01	0.30
Herbamix	0.15	0.00	0.15	0.00	0.30
Aflatoxin	25.55	0.00	24.95	0.14	50.64
Aflatoxin + Herbamix	24.91	0.27	23.93	1.42	50.52

Table 17. Calculated mycotoxin intake of laying hens per kg body weight

Group	Calculated total aflatoxin intake (µg/kg BW)				
	0-12 h	**12-24 h**	**24-36 h**	**36-48 h**	**0-48 h**
Control	0.07	0.00	0.07	0.01	0.15
Herbamix	0.08	0.00	0.07	0.00	0.15
Aflatoxin	13.16	0.00	12.83	0.08	26.07
Aflatoxin + Herbamix	13.40	0.15	11.98	0.70	26.23

Body weights, absolute and relative liver weights did not vary significantly during the experimental period (Table 18).

Table 18. Individual and combined effect of aflatoxin B_1 and medicinal herb mixture (Herbamix™) on body weight, liver weight and relative liver weight (mean±SD; n=5)

	Control	**Herbamix**	**Aflatoxin**	**Aflatoxin + Herbamix**
		Body weight (g)		
12th hour	1986.0± 116.4	1940.0± 149.5	1941.0± 155.2	1859.0± 100.8
24th hour	1819.0± 158.1	1782.0± 276.0	1924.0± 92.8	1817.0± 63.5
36th hour	2023.0± 178.5	2047.0± 228.5	1945.0± 183.0	1997.0± 128.0
48th hour	1806.0± 138.6	1853.0± 133.1	1768.0± 137.7	2029.0± 145.8
		Liver weight (g)		
12th hour	41.91± 6.59	39.85± 2.47	41.54± 3.58	42.88± 3.45
24th hour	34.22± 4.39	34.97± 8.08	41.57± 8.54	37.41± 2.86
36th hour	53.56± 2.98	48.69± 9.45	48.52± 4.49	48.80± 4.97
48th hour	38.22± 3.22	38.23± 7.66	35.51± 4.31	44.16± 8.11
		Relative liver weight (g/100 g body weight)		
12th hour	2.10± 0.24	2.06± 0.20	2.14± 0.10	2.31± 0.25
24th hour	1.89± 0.25	1.95± 0.20	2.16± 0.44	2.06± 0.17
36th hour	2.66± 0.27	2.37± 0.29	2.50± 0.14	2.44± 0.17
48th hour	2.12± 0.20	2.06± 0.35	2.01± 0.24	2.17± 0.30

Aflatoxin: 125 µg AFB_1/kg feed; Herbamix: 600 mg Herbamix Basic Premix/kg feed

5.2.1.1. Parameters of glutathione redox system and lipid peroxidation processes in blood plasma

Concentration of malondialdehyde (MDA), one of the end products of lipid peroxidation, showed significantly higher values at 12th hour sampling in aflatoxin treated group and in aflatoxin + herbal mixture treated group as compared to control (Table 19).

At hour 36 the aflatoxin + herbal mixture treatment also resulted significantly elevated MDA concentration as compared to control, while at hour 48 aflatoxin + herbal mixture treatment caused significantly elevated MDA concentrations as compared to the herbal mixture treated group (Table 19).

Table 19. Individual and combined effect of aflatoxin B_1 and medicinal herb mixture (Herbamix™) on some parameters of glutathione redox system and lipid peroxidation processes of blood plasma (mean±SD; n=5)

	Control	**Herbamix**	**Aflatoxin**	**Aflatoxin+Herbamix**
			MDA (µmol/L)	
12th hour	15.40[a]± 0.79	15.93[ab]± 1.51	18.48[bc]± 1.90	19.13[c]± 1.21
24th hour	19.53± 1.74	21.06± 2.78	21.14± 0.67	18.09± 0.84
36th hour	17.72[a]± 1.32	22.28[ab]± 5.04	25.24[ab]± 2.35	28.71[b]± 3.60
48th hour	26.07[b]± 2.11	20.69[a]± 0.52	23.27[ab]± 1.74	26.55[b]± 4.55
			GSH (µmol/g protein content)	
12th hour	3.43± 0.71	3.51± 0.73	3.71± 0.77	4.14± 0.93
24th hour	4.84± 1.07	3.95± 0.81	5.34± 2.09	4.89± 2.33
36th hour	2.83± 1.05	2.76± 0.74	2.42± 1.15	3.22± 1.81
48th hour	4.89± 1.87	5.18± 1.59	5.97± 1.22	6.33± 0.96
			GPx (U/g protein content)	
12th hour	4.60± 0.65	4.72± 1.55	5.07± 1.38	4.97± 1.71
24th hour	7.49± 1.79	5.66± 1.02	6.98± 1.88	6.93± 1.94
36th hour	6.93[b]± 1.94	4.17[a]± 0.98	3.82[a]± 0.28	4.04[a]± 0.98
48th hour	4.96± 0.51	4.61± 0.45	4.36± 1.08	5.14± 1.01

Aflatoxin: 125 µg AFB_1/kg feed; Herbamix: 600 mg Herbamix Basic Premix/kg feed

[a,b,c] Means designated with different letters within the same row mean significant difference ($p<0.05$)

No significant changes were found in reduced glutathione (GSH) concentration of blood plasma, while glutathione peroxidase (GPx) activity of blood plasma showed marked changes at hour 36.

At this sampling GPx activity of all treated groups was significantly higher than that of the control (Table 19).

5.2.1.2 Parameters of glutathione redox system and lipid peroxidation processes of red blood cell haemolysates

At 12th hour sampling malondialdehyde (MDA) concentration in RBC haemolysates of aflatoxin + herbal mixture treatment group was significantly lower as compared to the other experimental groups (Table 20), while at 36th hour sampling aflatoxin + herbal mixture treatment resulted significantly elevated MDA concentration as compared to control (Table 20).

Herbal mixture treatment caused significantly lower GSH concentration at hour 36 as compared to control group (Table 20).

At 48th hour sampling aflatoxin + herbal mixture treatment resulted significantly higher GPx activity as compared to the control or the herbal mixture treated group (Table 20).

Table 20. Individual and combined effect of aflatoxin B_1 and medicinal herb mixture (Herbamix™) on some parameters of glutathione redox system and lipid peroxidation processes of red blood cell haemolysates (mean±SD; n=5)

	Control	**Herbamix**	**Aflatoxin**	**Aflatoxin+Herbamix**
			MDA (µmol/L)	
12th hour	29.84[b]± 1.07	30.47[b]± 2.66	29.73[b]± 1.59	26.13[a]± 1.49
24th hour	38.57± 11.02	35.48± 7.03	37.32± 8.46	50.35± 14.13
36th hour	44.88[a]± 0.84	52.92[ab]± 5.58	45.32[ab]± 5.55	54.00[b]± 6.91
48th hour	44.16± 4.57	41.09± 3.77	34.07± 7.11	36.13± 12.14
			GSH (µmol/g protein content)	
12th hour	8.92± 0.82	7.23± 0.67	7.88± 1.34	7.90± 0.87
24th hour	6.91± 3.46	6.18± 2.05	5.16± 1.59	4.36± 2.41
36th hour	5.79[b]± 0.42	4.11[a]± 1.04	4.65[ab]± 0.40	4.87[ab]± 0.99
48th hour	4.43± 0.75	3.91± 1.25	6.41± 2.30	4.62± 0.70
			GPx (U/g protein content)	
12th hour	6.09± 1.01	6.25± 1.89	6.08± 0.79	6.05± 0.15
24th hour	5.55± 1.97	4.80± 1.19	4.04± 0.99	3.50± 1.03
36th hour	4.62± 0.56	4.14± 0.87	4.30± 0.46	4.24± 0.98
48th hour	4.15[a]± 0.71	4.14[a]± 0.81	5.47[ab]± 1.45	5.75[b]± 0.48

Aflatoxin: 125 µg AFB_1/kg feed; Herbamix: 600 mg Herbamix Basic Premix/kg feed

[a,b] Means designated with different letters within the same row mean significant difference ($p<0.05$)

5.2.1.3. Parameters of glutathione redox system and lipid peroxidation processes of tissue homogenates

As effect of aflatoxin, level of conjugated dienes and conjugated trienes - the initial phase markers of lipid peroxidation processes - did not change significantly in liver, and it was not modified by the supplementation of herbal mixture (Table 21).

Table 21. Individual and combined effect of aflatoxin and herbal product on conjugated diene (CD) and conjugated triene (CT) levels in liver (mean±SD; n=5)

	Control	**Herbamix**	**Aflatoxin**	**Aflatoxin+ Herbamix**
			CD (OD 232nm)	
12th hour	0.37± 0.16	0.33± 0.09	0.29± 0.03	0.30± 0.03
24th hour	0.29± 0.01	0.30± 0.08	0.33± 0.14	0.38± 0.11
36th hour	0.33± 0.07	0.31± 0.05	0.30± 0.07	0.27± 0.02
48th hour	0.28± 0.07	0.28± 0.04	0.33± 0.09	0.25± 0.02
			CT (OD 268nm)	
12th hour	0.20± 0.03	0.19± 0.02	0.18± 0.02	0.19± 0.01
24th hour	0.19± 0.01	0.17± 0.01	0.20± 0.03	0.19± 0.01
36th hour	0.16± 0.01	0.16± 0.01	0.15± 0.02	0.15± 0.01
48th hour	0.14± 0.02	0.15± 0.01	0.15± 0.02	0.13± 0.01

Aflatoxin: 125 µg AFB_1/kg feed; Herbamix: 600 mg Herbamix Basic Premix/kg feed

In terminal phase of lipid peroxidation processes, as described with malondialdehyde concentration, marked changes were noticed in the liver at hour 36 (Table 8). Single medicinal herb mixture resulted significantly higher MDA concentration as compared to aflatoxin + herbal mixture treatment (Table 22). Similar results were found in kidney samples at hour 48, where aflatoxin + herbal mixture treatment resulted significantly lower MDA values as compared to the herbal mixture treated group or to the control group (Table 23).

Table 22. Individual and combined effect of aflatoxin B_1 and medicinal herb mixture (Herbamix™) on some parameters of glutathione redox system and lipid peroxidation of liver homogenates (mean±SD; n=5)

	Control	**Herbamix**	**Aflatoxin**	**Aflatoxin+Herbamix**
		MDA (µmol/g wet weight)		
12th hour	8.28± 1.12	8.37± 2.41	10.59± 1.34	8.33± 1.68
24th hour	8.01± 2.66	7.21± 4.24	7.32± 2.96	6.76± 1.54
36th hour	13.61^{ab}± 1.74	14.28^{b}± 2.39	11.68^{ab}± 1.59	10.70^{a}± 1.65
48th hour	8.67± 2.02	7.60± 2.00	9.31± 0.46	9.83± 0.72
		GSH (µmol/g protein content of 10,000 g supernatant)		
12th hour	4.66± 1.20	4.64± 0.76	5.25± 1.87	6.06± 2.22
24th hour	3.78± 0.68	3.83± 0.93	3.17± 0.53	4.00± 0.71
36th hour	6.84± 0.68	7.79± 0.83	7.28± 0.41	6.61± 1.72
48th hour	5.25± 0.87	5.05± 0.44	4.46± 0.59	5.36± 1.03
		GPx (U/g protein content of 10,000 g supernatant)		
12th hour	4.22± 0.91	4.57± 0.83	5.13± 1.80	5.74± 2.20
24th hour	3.41± 0.88	3.75± 0.86	3.28± 0.49	4.01± 0.56
36th hour	7.01± 0.70	7.30± 0.50	7.04± 0.55	6.61± 1.77
48th hour	5.04± 0.73	4.99± 0.61	4.67± 0.58	5.51± 0.96

Aflatoxin: 125 µg AFB_1/kg feed; Herbamix: 600 mg Herbamix Basic Premix/kg feed

^{a,b} Means designated with different letters within the same row mean significant difference ($p<0.05$)

No significant changes were found in reduced glutathione (GSH) concentration and in GPx activity of liver and kidney homogenates (Table 22 and Table 23).

Table 23. Individual and combined effect of aflatoxin B_1 and medicinal herb mixture (Herbamix™) on some parameters of glutathione redox system and lipid peroxidation of kidney homogenates (mean±SD; n=5)

	Control	**Herbamix**	**Aflatoxin**	**Aflatoxin+Herbamix**
			MDA (µmol/g wet weight)	
12th hour	7.50± 1.58	9.84± 2.76	10.10± 1.07	10.29± 2.73
24th hour	12.44± 2.82	15.98± 3.46	19.36± 7.25	12.97± 2.56
36th hour	15.40± 2.86	11.57± 2.39	13.26± 2.50	15.28± 4.01
48th hour	9.80^{b}± 1.30	9.93^{b}± 1.50	9.15^{ab}± 2.28	6.72^{a}± 0.57
			GSH (µmol/g protein content of 10,000 g supernatant)	
12th hour	5.29± 0.42	5.19± 0.66	5.79± 0.62	5.24± 0.31
24th hour	6.08± 1.15	4.58± 1.53	5.81± 0.89	6.11± 0.40
36th hour	6.70± 0.31	7.08± 0.86	6.83± 0.50	7.55± 1.84
48th hour	5.75± 0.66	5.36± 0.73	4.74± 0.53	4.92± 0.43
			GPx (U/g protein content of 10,000 g supernatant)	
12th hour	5.17± 0.44	5.62± 0.60	6.39± 1.13	6.06± 0.43
24th hour	4.79± 0.78	3.96± 0.94	4.81± 0.83	4.94± 0.34
36th hour	4.67± 0.68	5.14± 0.41	5.49± 0.59	5.55± 0.53
48th hour	5.94± 0.60	6.11± 0.41	5.42± 1.01	5.96± 0.47

Aflatoxin: 125 µg AFB_1/kg feed; Herbamix: 600 mg Herbamix Basic Premix/kg feed

5.2.2. Discussion

The result of present study showed that short-term aflatoxin B_1 exposure had no effect on the feed intake, absolute and relative liver weight, probably due to the short period of investigation. In contrast with this finding, in long-term experiments with laying hens, AFB_1 caused marked feed refusal and increased liver weight (Aly Salwa and Anwer, 2009). However, AFB_1, even after short-term exposure (12 hours), has induced lipid peroxidation, which was confirmed by significant increase in the meta-stable end-product parameter (MDA) in the blood plasma and red blood cell haemolysate, but not in the liver and kidney. In the following 36 hours period of the experiment, MDA content in the AFB_1 treated group was nearly the same as in the control, which suggests that after a marked AFB_1-induced increase of free radical formation occurred, the antioxidant system activated and thus the further chain reaction of free radical-initiated lipid peroxidation was inhibited. In trials with long-term aflatoxin B_1 exposure much higher increase of MDA formation was found (Surai, 2002), which can explained by the depletion of antioxidant defense during a long period of time.

The combination of aflatoxin B_1 and medicinal herb mixture caused lower oxygen free radical formation in the liver and kidney resulting in lower lipid peroxidation rate and in reduced MDA concentration.

Among the parameters of the glutathione redox system in liver, GSH content and GPx activity did not show significant difference between the groups during the trial. These findings mean that both the actual GSH content and GPx activity in the liver were adequate for the elimination of aflatoxin-induced oxygen free radicals, or an activation happened between the 12^{th} and 24^{th} hours of mycotoxin exposure, since GPx is under allosteric regulation, which makes quick formation changes in the enzyme structure and activity (Pan et al., 2017). GSH and GPx have primary role in defense against oxygen free radicals, because they react with reactive oxygen species (ROS), degrade hydrogen peroxide and lipid peroxides (Pace et al., 2003). The slight insignificant changes revealed in GPx activity is contradictory with a previous finding where AFB_1 exposure caused significant drop in GPx activity in liver. However, this was revealed in a long-term trial (Shi et al., 2012), and the toxin possibly impaired both the *de novo* synthesis, and post-translational activation of GPx.

Within the parameters of the glutathione redox system, the GSH concentration changed only in the red blood cell haemolysate, as it was administered with medicinal herb mixture resulting in lower value than in the other groups. The main reason of this difference is not known; however, it might be explained by the difference in glutathione synthesis in the red blood cells.

This is a vital finding because erythrocyte glutathione plays important function in mitigating the detrimental effects of reactive oxygen species (ROS) present in the circulation (Mak et al., 1994); this causes continuous oxidation of hemoglobin within the cytoplasm of the erythrocyte (Hsieh and Jaffe, 1975). The activity of glutathione peroxidase did not vary in red blood cell haemolysate, liver and kidney, but there was significant difference in blood plasma of the treated groups.

According to the results it can be concluded that high level of aflatoxin induced ROS formation, and possessed mild oxidative stress, which was effectively eliminated by the glutathione redox system. This result suggests that if the amount and/or activity of glutathione system is adequate, high dose of aflatoxin induces only mild oxidative stress in the liver of laying hens within short period of time. However, during the short period of the study the positive effect of medicinal herb mixture (Herbamix™) was not confirmed at the applied dose.

5.3. Effects of a medicinal herb mixture pre-treatment and short-term T-2 toxin exposure in broiler chickens

5.3.1. Results

Consumption of high T-2/HT-2 toxin contaminated diet (which was approximately 4.52 times higher than the EU recommendation) did not cause mortality during the short-term trial, and no clinical signs of toxicity were observed.

Calculated feed intake showed some differences at different periods of the trial, but as feed intake was measured only for groups, no statistical analysis could be done on this parameter (Table 24).

Table 24. Individual and combined effect of T-2/HT-2 toxin and medicinal herb mixture (Herbamix™) on calculated feed intake of broiler chickens

Experimental group	Calculated feed intake per broiler chicken (g/bird)				
	0-12 h	**12-24 h**	**24-36 h**	**36-48 h**	**0-48 h**
Control	77.71	66.57	66.69	62.65	273.62
T-2 toxin	72.93	68.32	70.21	62.63	274.09
H1 + T-2 toxin	70.36	57.05	55.72	52.10	235.22
H2 + T-2 toxin	66.23	59.55	54.09	52.70	232.57
H5 + T-2 toxin	65.34	55.84	60.06	54.90	236.14

T-2 toxin: 1.13 mg T-2 toxin/kg feed and <0.1 mg HT-2 toxin/kg feed; H1: 300 mg Herbamix Basic Premix/kg feed; H2: 600 mg Herbamix Basic Premix/kg feed; H5: 1500 mg Herbamix Basic Premix/kg feed

Mycotoxin intake, which was calculated from feed intake and measured mycotoxin content of the particular complete feed, was almost the same among the groups fed with mycotoxin contaminated diet and mycotoxin contaminated and herbal mixture supplemented diets (Table 25 and 26).

Table 25. Calculated mycotoxin intake of broiler chickens per bird

Group	Calculated mycotoxin intake (μg/bird)									
	0-12 h		**12-24 h**		**24-36 h**		**36-48 h**		**0-48 h**	
	T-2 toxin	*HT-2 toxin*	T-2 toxin	*HT-2 toxin*	T-2 toxin	*HT-2 toxin*	T-2 toxin	*HT-2 toxin*	T-2 toxin	*HT-2 toxin*
Control	0.0008		0.0007		0.0007		0.0006		0.0027	
T-2 toxin	0.0824	*0.0007*	0.0772	*0.0007*	0.0793	*0.0007*	0.0708	*0.0006*	0.3097	*0.0027*
H1 + T-2 toxin	0.0795	*0.0007*	0.0645	*0.0006*	0.0630	*0.0006*	0.0589	*0.0005*	0.2658	*0.0024*
H2 + T-2 toxin	0.0748	*0.0007*	0.0673	*0.0006*	0.0611	*0.0005*	0.0596	*0.0005*	0.2658	*0.0023*
H5 + T-2 toxin	0.0738	*0.0007*	0.0631	*0.0006*	0.0679	*0.0006*	0.0620	*0.0005*	0.2668	*0.0024*

T-2 toxin: 1.13 mg T-2 toxin/kg feed and <0.1 mg HT-2 toxin/kg feed; H1: 300 mg Herbamix Basic Premix/kg feed; H2: 600 mg Herbamix Basic Premix/kg feed; H5: 1500 mg Herbamix Basic Premix/kg feed

Table 26. Calculated mycotoxin intake of broiler chickens per kg body weight

Group	Calculated mycotoxin intake (μg/kg BW)									
	0-12 h		**12-24 h**		**24-36 h**		**36-48 h**		**0-48 h**	
	T-2 toxin	*HT-2 toxin*	T-2 toxin	*HT-2 toxin*	T-2 toxin	*HT-2 toxin*	T-2 toxin	*HT-2 toxin*	T-2 toxin	*HT-2 toxin*
Control	1.03		0.85		0.90		0.74		3.52	
T-2 toxin	117.93	*1.04*	87.50	*0.77*	93.66	*0.83*	82.93	*0.73*	308.02	*3.38*
H1 + T-2 toxin	110.88	*0.98*	75.46	*0.67*	73.80	*0.65*	73.00	*0.65*	333.14	*2.95*
H2 + T-2 toxin	95.28	*0.84*	85.90	*0.76*	79.32	*0.70*	74.29	*0.66*	334.79	*2.96*
H5 + T-2 toxin	100.80	*0.89*	83.05	*0.73*	85.51	*0.76*	73.88	*0.65*	343.24	*3.04*

T-2 toxin: 1.13 mg T-2 toxin/kg feed and <0.1 mg HT-2 toxin/kg feed; H1: 300 mg Herbamix Basic Premix/kg feed; H2: 600 mg Herbamix Basic Premix/kg feed; H5: 1500 mg Herbamix Basic Premix/kg feed

Neither body weights nor absolute and relative liver weights showed significant changes during the trial (Table 27).

Table 27. Individual and combined effect of T-2/HT-2 toxin and different doses of medicinal herb mixture (Herbamix™) on body weight and on absolute and relative liver weight (mean±SD; n=6)

	CONTROL	T-2 TOXIN	H1 ± T-2 TOXIN	H2 ± T-2 TOXIN	H5 ± T-2 TOXIN
Body weight (g)					
12th hour	754.80± 69.16	698.80± 98.62	717.00± 94.59	785.50± 124.60	732.50± 62.10
24th hour	779.20± 66.01	882.30± 82.36	854.20± 46.81	783.30± 81.74	759.80± 108.30
36th hour	742.90± 94.01	847.10± 115.60	853.20± 73.43	770.60± 111.10	793.70± 68.34
48th hour	843.30± 88.49	853.30± 30.33	806.50± 92.96	801.60± 37.88	839.70± 103.30
Liver weight (g)					
12th hour	27.24± 3.37	21.14± 2.60	22.50± 4.83	24.03± 4.51	22.79 3.32
24th hour	23.93± 6.12	25.88± 2.96	28.36± 4.57	24.77± 5.56	24.63± 5.72
36th hour	21.16± 5.05	24.33± 8.21	22.11± 3.67	20.77± 4.33	22.84± 3.85
48th hour	21.55± 3.06	19.33± 2.95	19.38± 4.28	20.05± 3.43	20.63± 3.66
Relative liver weight (g /100 g body weight)					
12th hour	3.61± 0.29	3.04± 0.26	3.11± 0.35	3.05± 0.12	3.10± 0.30
24th hour	3.04± 0.52	2.94± 0.28	3.31± 0.41	3.13± 0.47	3.22± 0.49
36th hour	2.82± 0.40	2.82± 0.58	2.58± 0.23	2.68± 0.28	2.88± 0.43
48th hour	2.56± 0.30	2.27± 0.34	2.39± 0.33	2.50± 0.43	2.45± 0.23

T-2 toxin: 1.13 mg T-2 toxin/kg feed and <0.1 mg HT-2 toxin/kg feed; H1: 300 mg Herbamix Basic Premix/kg feed; H2: 600 mg Herbamix Basic Premix/kg feed; H5: 1500 mg Herbamix Basic Premix/kg feed

5.3.1.1. Parameters of glutathione redox system and lipid peroxidation processes in blood samples

In RBC haemolysates of T-2/HT-2 toxin treated group, concentration of MDA (one of the end products of lipid peroxidation processes) increased significantly ($p<0.05$) at 12h as compared to control (Table 29). Herbamix treatment had beneficial effect resulting in lower MDA concentration at 36h both in blood plasma and RBC haemolysate as compared to T-2/HT-2 toxin alone (Table 29 and 28).

As effect of T-2/HT-2 toxin treatment the GSH concentration of plasma increased moderately at 12h and 24h compared to control (Table 28), while significant increase was measured in plasma and also in RBC haemolysate at 24h in T-2/HT-2 toxin + highest Herbamix dose group (Table 28 and 29).
GPx activity of plasma was also increased ($p<0.05$) by the T-2/HT-2 toxin treatment at 12h (Table 1), while most marked ($p<0.05$) elevations were found in T-2/HT-2 toxin + medium Herbamix dose group in plasma at 12h and 36h (Table 28), and in RBC haemolysate at 24h (Table 29).

Table 28. Individual and combined effect of T-2/HT-2 toxin and different doses of medicinal herb mixture (Herbamix™) on some parameters of glutathione redox system and lipid peroxidation processes of blood plasma (mean±SD; n=6)

	CONTROL	T-2 TOXIN	H1 + T-2 TOXIN	H2 + T-2 TOXIN	H5 + T-2 TOXIN
Malondialdehyde (MDA) (μmol/L)					
12th hour	4.22± 0.68	3.72± 0.41	4.43± 0.77	3.92± 1.10	4.35± 0.46
24th hour	2.99^{a}± 1.48	3.26^{ab}± 1.09	4.90^{b}± 0.60	4.20^{ab}± 0.93	3.84^{ab}± 0.98
36th hour	4.75^{ab}± 2.32	4.92^{b}± 1.36	3.47^{ab}± 1.33	4.05^{ab}± 1.10	2.44^{a}± 0.62
48th hour	3.43^{a}± 1.03	3.08^{a}± 1.56	4.32^{ab}± 0.46	3.80^{a}± 0.86	6.09^{b}± 1.29
Reduced glutathione (GSH) (μmol/g protein content)					
12th hour	10.28^{ab}± 0.81	11.86^{b}± 2.19	9.64^{ab}± 1.29	10.79^{ab}± 1.85	8.95^{a}± 0.69
24th hour	7.95^{a}± 0.99	9.27^{ab}± 2.00	8.29^{ab}± 1.40	10.18^{ab}± 1.35	10.41^{b}± 1.69
36th hour	10.21^{ab}± 0.79	9.07^{ab}± 1.60	9.22^{ab}± 1.80	11.16^{b}± 1.37	7.90^{a}± 1.17
48th hour	6.91± 0.56	5.69± 1.17	5.98± 1.06	5.42± 0.41	5.87± 1.33
Glutathione peroxidase (GPx) (U/g protein content)					
12th hour	5.16^{a}± 0.37	8.50^{b}± 1.47	6.51^{a}± 1.24	9.29^{bc}± 1.32	6.88^{ab}± 0.88
24th hour	8.24^{b}± 0.66	8.07^{b}± 1.25	6.02^{a}± 1.11	6.88^{ab}± 1.58	7.78^{ab}± 1.32
36th hour	7.71^{a}± 1.99	8.70^{ab}± 0.82	8.00^{a}± 1.73	10.90^{b}± 1.12	9.15^{ab}± 1.99
48th hour	7.10± 1.15	6.26± 1.36	5.76± 0.96	6.51± 0.64	6.25± 1.23

T-2 toxin: 1.13 mg T-2 toxin/kg feed and <0.1 mg HT-2 toxin/kg feed; H1: 300 mg Herbamix Basic Premix/kg feed; H2: 600 mg Herbamix Basic Premix/kg feed; H5: 1500 mg Herbamix Basic Premix/kg feed

^{a,b} Means designated with different letters within the same row mean significant difference ($p<0.05$)

Table 29. Individual and combined effect of T-2/HT-2 toxin and different doses of medicinal herb mixture (Herbamix™) on some parameters of glutathione redox system and lipid peroxidation processes of red blood cell hemolysate (mean±SD; n=6)

	CONTROL	T-2 TOXIN	H1 + T-2 TOXIN	H2 + T-2 TOXIN	H5 + T-2 TOXIN
Malondialdehyde (MDA) (μmol/L)					
12th hour	6.46^{a}± 1.30	8.67^{b}± 1.10	7.91^{ab}± 0.61	8.37^{b}± 0.95	7.44^{ab}± 0.48
24th hour	9.25± 0.67	7.82± 0.71	8.71± 1.02	8.65± 0.53	7.83± 2.46
36th hour	7.89^{ab}± 0.68	8.44^{b}± 1.50	6.46^{a}± 1.37	7.03^{ab}± 1.36	6.27^{a}± 0.71
48th hour	11.93± 1.23	10.55± 2.59	10.14± 2.12	10.82± 2.96	8.57± 1.93
Reduced glutathione (GSH) (μmol/g protein content)					
12th hour	13.49± 2.60	12.10± 1.12	13.61± 2.79	12.89± 2.56	12.26± 1.26
24th hour	11.84^{a}± 1.60	11.70^{a}± 3.29	11.94^{a}± 1.72	15.35^{ab}± 1.96	17.68^{b}± 2.09
36th hour	15.23± 3.14	12.66± 3.98	13.33± 2.98	12.87± 1.55	13.04± 2.41
48th hour	10.64± 3.01	9.86± 2.69	9.61± 2.59	11.00± 4.01	8.62± 3.09
Glutathione peroxidase (GPx) (U/g protein content)					
12th hour	6.95± 1.08	5.79± 0.49	6.59± 1.43	5.88± 1.33	5.70± 0.52
24th hour	5.30^{a}± 0.94	5.42^{a}± 1.34	5.63^{ab}± 0.99	7.46^{bc}± 1.12	8.29^{c}± 1.27
36th hour	6.85± 1.15	6.67± 0.90	7.03± 1.77	5.34± 1.59	6.45± 1.28
48th hour	5.72± 0.75	5.41± 1.00	5.68± 1.06	5.48± 0.82	5.89± 1.19

T-2 toxin: 1.13 mg T-2 toxin/kg feed and <0.1 mg HT-2 toxin/kg feed; H1: 300 mg Herbamix Basic Premix/kg feed; H2: 600 mg Herbamix Basic Premix/kg feed; H5: 1500 mg Herbamix Basic Premix/kg feed

^{a,b,c} Means designated with different letters within the same row mean significant difference ($p<0.05$)

5.3.1.2. Parameters of glutathione redox system and lipid peroxidation processes in tissue homogenates

As effect of T-2/HT-2 toxin alone, level of conjugated dienes and conjugated trienes, the initial phase markers of lipid peroxidation processes, did not change significantly in liver, while in combination with herbal mixture significantly lower values were measured at 24h in T-2/HT-2 toxin + medium Herbamix dose group as compared to control and to T-2/HT-2 toxin treated group (Table 30). At 36h in T-2/HT-2 toxin + highest Herbamix dose group also significantly lower CD and CT values were measured than in control and in T-2/HT-2 toxin treated group (Table 30)

Table 30. Individual and combined effect of T-2/HT-2 toxin and different doses of medicinal herb mixture (Herbamix™) on conjugated diene (CD) and conjugated triene (CT) levels of liver homogenates (mean±SD; n=6)

	CONTROL	**T-2 TOXIN**	**H1 + T-2 TOXIN**	**H2 + T-2 TOXIN**	**H5 + T-2 TOXIN**
Conjugated dienes (CD) (OD 232 nm)					
12th hour	0.743± 0.064	0.732± 0.051	0.788± 0.048	0.771± 0.100	0.792± 0.086
24th hour	0.622^{b}± 0.038	0.622^{b}± 0.036	0.634^{b}± 0.048	0.550^{a}± 0.042	0.595^{ab}± 0.038
36th hour	0.722^{b}± 0.076	0.714^{b}± 0.064	0.600^{a}± 0.040	0.564^{a}± 0.049	0.532^{a}± 0.076
48th hour	0.612± 0.077	0.618± 0.031	0.653± 0.066	0.628± 0.042	0.626± 0.045
Conjugated trienes (CT) (OD 268 nm)					
12th hour	0.230± 0.016	0.234± 0.010	0.241± 0.009	0.227± 0.026	0.235± 0.020
24th hour	0.224^{b}± 0.009	0.218^{b}± 0.015	0.219^{b}± 0.012	0.191^{a}± 0.011	0.211^{ab}± 0.010
36th hour	0.214^{bc}± 0.021	0.217^{c}± 0.009	0.204^{abc}± 0.013	0.192^{ab}± 0.007	0.184^{a}± 0.013
48th hour	0.221± 0.019	0.216± 0.013	0.233± 0.018	0.215± 0.009	0.221± 0.013

T-2 toxin: 1.13 mg T-2 toxin/kg feed and <0.1 mg HT-2 toxin/kg feed; H1: 300 mg Herbamix Basic Premix/kg feed; H2: 600 mg Herbamix Basic Premix/kg feed; H5: 1500 mg Herbamix Basic Premix/kg feed

^{a,b,c} Means designated with different letters within the same row mean significant difference ($p<0.05$)

In liver homogenates, concentration of MDA, the meta-stable end product of lipid peroxidation processes, was markedly higher at 24h, and significantly higher at 48h in T-2/HT-2 toxin treated group as compared to control (Table 31), while the highest dose of Herbamix treatment had beneficial effect resulting in lower MDA concentration at 24h and 48h of mycotoxin exposure as compared to T-2/HT-2 toxin alone (Table 31).

GSH concentration and GPx activity in liver homogenate showed differences at 24 hour sampling, out of which T-2/HT-2 toxin + medium and highest dose of herbal mixture combination caused significantly lower values than in T-2/HT-2 toxin treated group (Table 31). At 36h significantly higher GSH concentrations were measured in T-2/HT-2 toxin + lowest and highest dose herbal mixture groups as compared to control (Table 31).

In kidney homogenates, MDA concentration was significantly higher at 48h in T-2/HT-2 + medium dose of Herbamix treatment group as compared to the T-2/HT-2 toxin treated one (Table 32).

In T-2/HT-2 + medium dose of Herbamix treatment group the GSH concentration at 12h significantly exceeded the values measured in control and in T-2/HT-2 toxin treated group (Table 32).

Similarly to the changes of its co-substrate, the GPx activity showed significantly higher values in kidney homogenates in T-2/HT-2 + medium dose of Herbamix treatment group at 12h, 36h and 48h of mycotoxin exposure compared to T-2/HT-2 toxin treated group or to the control group (Table 32).

Table 31. Individual and combined effect of T-2/HT-2 toxin and different doses of medicinal herb mixture (Herbamix™) on some parameters of glutathione redox system and lipid peroxidation processes of liver homogenates (mean±SD; n=6)

	CONTROL	T-2 TOXIN	H1 + T-2 TOXIN	H2 + T-2 TOXIN	H5 + T-2 TOXIN
Malondialdehyde (MDA) (μmol/g wet weight)					
12th hour	4.44± 1.40	3.54± 1.01	3.45± 0.95	4.46± 2.08	3.42± 1.51
24th hour	4.12^{abc}± 0.42	5.59^{c}± 1.27	3.62^{ab}± 1.48	5.05^{bc}± 0.46	3.27^{a}± 0.78
36th hour	3.39± 1.39	3.18± 0.71	4.74± 0.89	4.39± 1.82	3.52± 1.19
48th hour	3.21^{a}± 0.44	5.61^{b}± 1.23	3.96^{a}± 0.45	5.72^{b}± 1.38	3.37^{a}± 0.93
Reduced glutathione (GSH) (μmol/g protein content of 10,000 g supernatant)					
12th hour	4.84± 0.41	5.40± 0.32	4.83± 1.05	5.42± 0.77	4.63± 0.81
24th hour	4.16^{ab}± 0.75	4.65^{b}± 0.85	4.26^{ab}± 0.72	3.34^{a}± 0.40	3.20^{a}± 0.99
36th hour	4.26^{a}± 0.70	5.73^{ab}± 2.10	6.98^{b}± 1.27	6.54^{ab}± 1.85	7.07^{b}± 0.75
48th hour	3.83± 0.51	4.29± 0.60	3.94± 0.73	4.40± 0.42	3.71± 0.30
Glutathione peroxidase (GPx) (U/g protein content of 10,000 g supernatant)					
12th hour	4.54± 0.30	4.83± 0.40	4.48± 0.95	4.81± 0.60	4.13± 0.64
24th hour	3.93^{ab}± 0.78	4.72^{b}± 0.78	4.19^{ab}± 0.63	3.22^{a}± 0.30	2.94^{a}± 1.08
36th hour	3.75^{a}± 0.87	5.06^{ab}± 2.40	6.78^{b}± 1.15	6.34^{ab}± 1.68	6.57^{b}± 0.67
48th hour	3.94± 0.86	4.73± 0.64	4.75± 0.94	5.16± 0.54	4.67± 0.44

T-2 toxin: 1.13 mg T-2 toxin/kg feed and <0.1 mg HT-2 toxin/kg feed; H1: 300 mg Herbamix Basic Premix/kg feed; H2: 600 mg Herbamix Basic Premix/kg feed; H5: 1500 mg Herbamix Basic Premix/kg feed

^{a,b} Means designated with different letters within the same row mean significant difference ($p<0.05$)

Table 32. Individual and combined effect of T-2/HT-2 toxin and different doses of medicinal herb mixture (Herbamix™) on some parameters of glutathione redox system and lipid peroxidation processes of kidney homogenates (mean±SD; n=6)

	CONTROL	T-2 TOXIN	H1 + T-2 TOXIN	H2 + T-2 TOXIN	H5 + T-2 TOXIN
Malondialdehyde (MDA) (μmol/g wet weight)					
12th hour	4.18± 1.62	4.75± 2.00	3.25± 1.09	4.93± 0.65	4.17± 1.34
24th hour	6.80± 3.51	6.03± 4.17	4.90± 1.19	4.48± 3.45	6.01± 2.98
36th hour	5.47± 2.93	3.48± 1.48	4.78± 2.46	4.30± 1.47	5.19± 3.80
48th hour	3.24^{ab}± 1.05	1.95^{a}± 0.74	3.53^{ab}± 1.20	4.29^{b}± 0.36	3.14^{ab}± 1.37
Reduced glutathione (GSH) (μmol/g protein content of 10,000 g supernatant)					
12th hour	4.23^{a}± 0.68	4.19^{a}± 0.81	3.88^{a}± 0.95	5.66^{b}± 0.99	5.14^{ab}± 0.51
24th hour	6.20± 0.70	5.34± 1.04	5.51± 0.48	6.20± 1.21	5.28± 0.75
36th hour	3.66± 1.00	4.05± 1.05	4.82± 1.37	5.12± 0.89	3.95± 0.61
48th hour	3.93± 0.86	3.46± 0.58	4.07± 0.90	4.44± 0.74	4.52± 0.79
Glutathione peroxidase (GPx) (U/g protein content of 10,000 g supernatant)					
12th hour	3.39^{ab}± 1.87	2.85^{a}± 0.48	3.09^{ab}± 0.99	4.78^{b}± 0.67	4.37^{ab}± 0.70
24th hour	5.21± 0.57	4.70± 1.45	5.22± 0.66	5.52± 0.77	4.34± 0.71
36th hour	3.05^{a}± 0.50	3.31^{ab}± 0.82	4.23^{ab}± 1.22	4.36^{b}± 0.87	3.17^{a}± 0.39
48th hour	2.29^{a}± 0.37	2.33^{a}± 0.56	3.71^{b}± 1.07	4.22^{b}± 0.65	4.56^{b}± 1.06

T-2 toxin: 1.13 mg T-2 toxin/kg feed and <0.1 mg HT-2 toxin/kg feed; H1: 300 mg Herbamix Basic Premix/kg feed; H2: 600 mg Herbamix Basic Premix/kg feed; H5: 1500 mg Herbamix Basic Premix/kg feed

^{a,b} Means designated with different letters within the same row mean significant difference ($p<0.05$)

5.3.1.3. Clinical biochemical parameters in blood plasma

In case of T-2/HT-2 toxin + highest dose herbal mixture combination aspartate-aminotransferase (AST) and gamma-glutamyltransferase (GGT) activities of blood plasma showed significantly lower values as compared to the control at 24h and 48h samplings, respectively (Table 33). Among the measured clinical biological parameters of the blood plasma glucose concentrations were significantly higher in case of T-2/HT-2 toxin + lowest and highest dose herbal mixture combination as compared to control (Table 33). Uric acid concentration measured at 24h sampling was significantly higher in control group than in the other experimental treated groups (Table 33). In cholesterol concentrations of blood plasma samples no differences were found, but plasma triglycerides showed marked elevation ($p<0.05$) in T-2/HT-2 toxin treated group and in T-2/HT-2 toxin + highest dose of herbal mixture combination as compared to T-2/HT-2 toxin + lowest dose of herbal mixture group (Table 33).

Table 33. Individual and combined effect of T-2/HT-2 toxin and different doses of medicinal herb mixture (Herbamix™) on some clinical chemical parameters of blood plasma (mean±SD; n=6)

	CONTROL	**T-2 TOXIN**	**H1 + T-2 TOXIN**	**H2 + T-2 TOXIN**	**H5 + T-2 TOXIN**
Aspartate aminotransferase (AST) (U/l)					
12th hour	144.90± 10.34	160.30± 14.11	157.10± 21.78	150.20± 20.75	164.50± 6.77
24th hour	172.00^{b}± 23.69	165.30^{ab}± 40.26	139.30^{ab}± 54.25	126.30^{ab}± 17.66	115.50^{a}± 19.52
36th hour	120.60± 14.13	128.90± 27.47	131.50± 15.44	135.30± 9.90	131.20± 19.06
48th hour	157.80± 25.42	151.90± 9.49	150.50± 28.00	147.80± 22.17	141.30± 14.57
Gamma-glutamyltransferase (GGT) (U/l)					
12th hour	52.70± 20.87	54.82± 20.96	54.10± 34.87	44.25± 11.40	39.97± 13.67
24th hour	51.17± 17.68	53.83± 18.39	32.92± 7.94	40.53± 36.20	35.43± 14.78
36th hour	25.56± 4.82	30.86± 14.43	26.98± 7.23	28.52± 4.93	31.06± 3.15
48th hour	52.51^{b}± 13.40	34.21^{a}± 7.76	41.26^{ab}± 12.23	39.12^{ab}± 8.82	34.71^{a}± 5.64

	CONTROL	T-2 TOXIN	H1 + T-2 TOXIN	H2 + T-2 TOXIN	H5 + T-2 TOXIN
			Glucose (mmol/l)		
12th hour	9.57± 1.57	8.72± 1.34	8.33± 1.10	7.75± 0.80	8.35± 0.35
24th hour	7.28^{a}± 0.75	8.18^{ab}± 0.51	8.92^{b}± 0.38	8.27^{ab}± 1.49	8.79^{b}± 0.83
36th hour	8.61± 0.82	8.63± 0.99	7.92± 0.49	8.00± 0.78	9.10± 0.53
48th hour	8.38± 0.65	7.99± 1.12	8.45± 0.90	7.69± 0.65	7.54± 0.60
			Cholesterol (mmol/l)		
12th hour	3.13± 0.31	2.53± 0.27	3.36± 0.27	3.08± 0.95	2.74± 0.34
24th hour	3.02± 0.29	3.11± 0.78	2.90± 0.45	3.23± 0.65	2.85± 0.56
36th hour	3.01± 0.78	3.31± 0.61	3.42± 0.24	3.43± 0.52	3.87± 0.33
48th hour	4.04± 0.39	3.77± 0.58	3.95± 0.83	3.77± 0.57	3.74± 0.34
			Triglycerides (mmol/l)		
12th hour	1.22± 0.38	1.44± 0.46	1.59± 0.98	1.69± 0.86	1.34± 0.31
24th hour	1.60± 0.53	1.65± 0.24	1.81± 0.25	1.93± 0.88	1.40± 0.49
36th hour	1.11^{ab}± 0.53	1.50^{b}± 0.44	0.80^{a}± 0.50	0.93^{ab}± 0.25	1.55^{b}± 0.06
48th hour	1.24± 0.63	1.53± 0.06	1.52± 0.21	1.49± 0.13	1.37± 0.09
			Uric acid (µmol/l)		
12th hour	556.40± 75.08	579.50± 81.26	685.70± 145.00	699.10± 130.20	570.00± 106.60
24th hour	871.30^{b}± 194.60	540.10^{a}± 130.20	430.00^{a}± 93.74	430.50^{a}± 73.68	440.80^{a}± 47.69
36th hour	467.50± 66.79	484.60± 127.00	339.50± 47.18	321.50± 60.19	710.90± 120.90
48th hour	872.80± 189.70	509.60± 123.30	648.60± 145.30	568.10± 102.90	449.30± 94.27

T-2 toxin: 1.13 mg T-2 toxin/kg feed and <0.1 mg HT-2 toxin/kg feed; H1: 300 mg Herbamix Basic Premix/kg feed; H2: 600 mg Herbamix Basic Premix/kg feed; H5: 1500 mg Herbamix Basic Premix/kg feed

^{a,b} Means designated with different letters within the same row mean significant difference ($p<0.05$)

5.3.2. Discussion

In the present experiment the contamination level of T-2/HT-2 toxin in feed was 2.25-fold than its tolerable level (0.5 mg/kg feed) for broilers as proposed by Eriksen and Pettersson (2004), and 4.5-fold as the proposed maximum level (0.25 mg/kg feed) of the European Commission (2013).

In a long-term experiment with broiler chickens Weber et al. (2006) investigated pathophysiological changes associated with the consumption of mycotoxin contaminated (2.35 mg T-2 toxin /kg feed) diet, which did not enhance the lipid peroxidation processes, but affected the glutathione redox system in blood plasma. Using lower mycotoxin contaminated diet (1.04 mg T-2 toxin /kg feed and 0.49 mg HT-2 toxin /kg feed) Weber et al. (2010) did not find any alteration in MDA concentration, GSH concentration and GPx activity of blood plasma during the starter phase of broiler chicken rearing.

In another long-term trial Balogh et al. (2015) also did not find significant changes in lipid peroxidation processes and in the glutathione redox system after feeding broiler chickens with experimentally contaminated (1.5 mg T-2 toxin /kg feed) diet for 28 days.

In contrary, Rezar et al. (2007) in an 18-days-long experiment done with 3 weeks old broilers did not find significant alterations in blood plasma MDA concentrations and GPx activity of erythrocytes in case of the applied doses (0.5, 1.5, 4.5 and 13.5 mg/kg feed) of T-2 toxin.

In a short-term experiment done by Bócsai et al. (2016), as a result of feeding almost 3-times higher T-2 toxin contaminated diet (3.09 mg T-2 toxin/kg) than in present study, the glutathione redox system activated shortly after the mycotoxin exposure is started, which is supported by the significantly higher concentration of reduced glutathione and glutathione peroxidase activity in blood plasma at 24h and 48h.

In case of T-2/HT-2 toxin + highest dose herbal mixture combination aspartate-aminotransferase (AST) and gamma-glutamyltransferase (GGT) activities of blood plasma were significantly lower as compared to the control at 24h and 48h samplings, respectively. These results are contradictory with some previous trials where increase of AST or GGT activity were found. For instance Yang et al. (2016) found that high dose of T-2/HT-2 toxin (4 mg/kg and 0.667 mg/kg) resulted in significantly higher AST activity in a long-term study. In case of GGT activity Manafi et al. (2012) found higher activity as effect of feeding T-2 toxin contaminated diet in broiler chicken. In a long-term (35 days long) experiment done with Japanese quail (*Coturnix coturnix japonica*) chicks Madheswaran et al. (2004) investigated the effect of feeding diet containing 4 mg/kg T-2 toxin. At the end of the experiment AST activity was significantly higher than in control, but no significant alterations were found in GGT activity. These results suggest that the applied herbal mixture supplementation has beneficial effect against liver damage caused by T-2/HT-2 toxin, as confirmed by the lower activity of liver-specific enzymes AST or GGT.

Blood plasma glucose concentrations were significantly higher in case of T-2/HT-2 toxin + lowest and highest dose herbal mixture combination as compared to control. This result is also contradictory of previous studies where high doses of T-2 toxin (4 mg/kg) fed from 7 to 49 days (Raina et al., 1991), and 6 and 8 mg/kg feed in the first 3 weeks (Kubena et al., 1994) resulted in reduced glucose levels.

The authors stated that hypoglycaemia could be attributed to the hepatic damage observed in toxin-fed birds. In the present study no such effect was found which suggests that herbal mixture combination prevents liver damage, therefore no hypoglycaemia was induced.

Uric acid concentration measured at 24h sampling was significantly higher in the control group than in the other experimental treated groups. This finding is contrary with the results of a 5-week-long trial done with broiler chickens, where serum uric acid levels were decreased significantly during T-2 toxicosis (Manafi et al., 2012). Arvind et al. (2003) reported also reduction in uric acid values in broilers receiving diets containing 5 mg/kg T-2 toxin. As it is the end product of protein metabolism, decreased uric acid level is presumably due to decreased feed consumption leading to decreased protein utilization and metabolism. Results of present study suggest that herbal mixture prevents the negative effect of T-2/HT-2 toxin on protein utilization and metabolism, and also on feed intake.

In cholesterol concentrations of blood plasma samples, no differences were found, while hypocholesterolaemia was reported in broiler chickens fed 6 and 8 mg T-2 toxin/kg from 0-3 weeks (Kubena et al., 1989; Kubena et al., 1994).

The results revealed that the applied trichothecene mycotoxin, T-2 toxin, had induced oxygen free radical formation, and consequently caused activation on the glutathione redox system redox system of broiler chickens, namely synthesis of reduced glutathione and glutathione peroxidase. Two-weeks pre-treatment of herbal mixture had only moderate effect against the mild oxidative stress caused by T-2 toxin at the dose applied, but it prevented liver damage and metabolic disturbances.

5.4. Effects of a medicinal herb mixture pre-treatment and short-term aflatoxin exposure in broiler chickens

5.4.1. Results

As a result of high aflatoxin intake (7 times higher AFB_1 concentration in feed than the EU regulation) there was no morbidity and mortality in the experimental groups during this short-term study, and no clinical signs of aflatoxicosis were observed.

Calculated feed intake showed some differences at different periods of the trial, but as feed intake was measured only for groups, no statistical analysis could be done on this parameter (Table 34).

Table 34. Individual and combined effect of aflatoxin and medicinal herb mixture (Herbamix™) on calculated feed intake of broiler chickens

Experimental group	Calculated feed intake per broiler chicken (g/bird)				
	0-12 h	**12-24 h**	**24-36 h**	**36-48 h**	**0-48 h**
Control	64.89	44.36	60.25	52.80	222.31
Aflatoxin	65.21	44.00	62.70	59.93	231.84
H1 + Aflatoxin	59.54	43.77	58.42	55.05	216.78
H2 + Aflatoxin	57.21	43.27	48.19	45.70	194.37
H5 + Aflatoxin	59.21	43.73	55.25	45.80	203.99

Aflatoxin: 140 µg AFB_1/kg feed; H1: 300 mg Herbamix Basic Premix/kg feed; H2: 600 mg Herbamix Basic Premix/kg feed; H5: 1500 mg Herbamix Basic Premix/kg feed

Mycotoxin intake, which was calculated from feed intake and measured mycotoxin content of the particular complete feed, was almost the same between the groups fed with mycotoxin contaminated diet and mycotoxin contaminated and herbal mixture supplemented diets (Table 35 and Table 36).

Table 35. Calculated total aflatoxin intake (µg/bird) of broiler chickens

Group	Calculated mycotoxin intake (µg/bird)									
	0-12 h		**12-24 h**		**24-36 h**		**36-48 h**		**0-48 h**	
	total AF	*in which AFB_1*	total AF	*in which AFB_1*	total AF	*in which AFB_1*	total AF	*in which AFB_1*	total AF	*in which AFB_1*
Control	0.078	*0.078*	0.053	*0.053*	0.072	*0.072*	0.063	*0.063*	0.267	*0.267*
Aflatoxin	9.763	*9.130*	6.589	*6.160*	9.386	*8.776*	8.971	*8.386*	34.706	*32.457*
H1 + Aflatoxin	8.912	*8.335*	6.553	*6.128*	8.745	*8.179*	8.241	*7.707*	32.451	*30.349*
H2 + Aflatoxin	8.565	*8.010*	6.478	*6.058*	7.218	*6.746*	6.841	*6.398*	29.098	*27.212*
H5 + Aflatoxin	8.864	*8.290*	6.546	*6.122*	8.271	*7.735*	6.856	*6.412*	30.537	*28.559*

Aflatoxin: 140 µg AFB_1/kg feed; H1: 300 mg Herbamix Basic Premix/kg feed; H2: 600 mg Herbamix Basic Premix/kg feed; H5: 1500 mg Herbamix Basic Premix/kg feed

Table 36. Calculated total aflatoxin intake of broiler chickens per kg body weight

Group	Calculated mycotoxin intake (µg/kg BW)									
	0-12 h		**12-24 h**		**24-36 h**		**36-48 h**		**0-48 h**	
	total AF	*in which AFB_1*	total AF	*in which AFB_1*	total AF	*in which AFB_1*	total AF	*in which AFB_1*	total AF	*in which AFB_1*
Control	0.11	*0.11*	0.08	*0.08*	0.09	*0.09*	0.09	*0.09*	0.37	*0.37*
Aflatoxin	14.57	*13.62*	8.81	*8.24*	13.16	*12.31*	12.00	*11.22*	48.53	*45.39*
H1 + Aflatoxin	13.20	*12.35*	9.85	*9.21*	12.24	*11.45*	11.96	*11.19*	47.26	*44.19*
H2 + Aflatoxin	13.90	*13.00*	8.84	*8.27*	9.87	*9.23*	9.03	*8.44*	41.63	*38.94*
H5 + Aflatoxin	13.35	*12.49*	9.43	*8.82*	10.20	*9.54*	9.87	*9.23*	42.86	*40.09*

Aflatoxin: 140 µg AFB_1/kg feed; H1: 300 mg Herbamix Basic Premix/kg feed; H2: 600 mg Herbamix Basic Premix/kg feed; H5: 1500 mg Herbamix Basic Premix/kg feed

Neither body weights nor absolute and relative liver weights showed significant changes during the trial, which is probably due to the short (48 hours long) period of investigation (Table 37).

Table 37. Individual and combined effect of aflatoxin and different doses of medicinal herb mixture (Herbamix™) on body weight and on absolute and relative liver weight (mean±SD; n=6)

	CONTROL	AFLATOXIN	H1 + AFLATOXIN	H2 + AFLATOXIN	H5 + AFLATOXIN
Body weight (g)					
12th hour	716.30± 96.67	670.20± 87.48	675.10± 52.67	616.30± 91.10	663.90± 93.64
24th hour	647.50± 103.70	748.00± 116.60	665.30± 47.66	732.80± 78.05	693.80± 69.51
36th hour	821.20± 57.62	713.10± 129.10	714.40± 75.32	730.90± 101.70	810.60± 61.15
48th hour	722.30± 84.22	747.80± 117.10	688.80± 70.08	757.80± 50.47	694.50± 62.46
Liver weight (g)					
12th hour	26.97± 4.89	23.40± 3.97	26.77± 4.85	21.60± 4.60	22.46± 4.55
24th hour	20.29± 3.16	24.18± 4.50	20.07± 2.72	23.94± 3.41	22.58± 4.05
36th hour	25.20± 1.88	22.39± 4.33	22.10± 2.96	20.70± 3.59	24.74± 2.97
48th hour	19.74± 2.37	20.20± 6.45	20.62± 4.66	19.37± 2.19	17.36± 2.10
Relative liver weight (g /100 g body weight)					
12th hour	3.76± 0.38	3.48± 0.29	3.96± 0.59	3.48± 0.29	3.37± 0.34
24th hour	3.15± 0.28	3.23± 0.33	3.02± 0.39	3.26± 0.29	3.25± 0.35
36th hour	3.08± 0.23	3.14± 0.09	3.09± 0.20	2.82± 0.23	3.05± 0.27
48th hour	2.73± 0.15	2.65± 0.46	2.97± 0.40	2.56± 0.30	2.50± 0.24

Aflatoxin: 140 µg AFB_1/kg feed; H1: 300 mg Herbamix Basic Premix/kg feed; H2: 600 mg Herbamix Basic Premix/kg feed; H5: 1500 mg Herbamix Basic Premix/kg feed

5.4.1.1. Parameters of glutathione redox system and lipid peroxidation processes in blood samples

MDA concentration in blood plasma of aflatoxin + highest Herbamix dose resulted significantly lower MDA concentration at 48h as compared to control (Table 38), while in RBC haemolysates of aflatoxin treated group it was increased ($p<0.05$) at 36h (Table 39).

At 24h, GSH concentration of blood plasma increased ($p<0.05$) in all experimental groups (Table 38), which suggests response of the organism to oxidative stress as provoked by aflatoxin exposure, while in RBC haemolysates the aflatoxin + medium Herbamix dose resulted significantly lower concentrations at 24h and 48h compared to control (Table 39).

GPx activity of blood plasma was significantly lower in aflatoxin treated group at 24h as compared to control (Table 38), while no significant changes were found in RBC haemolysates (Table 39).

Table 38. Individual and combined effect of aflatoxin and different doses of medicinal herb mixture (Herbamix™) on some parameters of glutathione redox system and lipid peroxidation processes of blood plasma (mean±SD; n=6)

	CONTROL	AFLATOXIN	H1 + AFLATOXIN	H2 + AFLATOXIN	H5 + AFLATOXIN
Malondialdehyde (MDA) (μmol/L)					
12th hour	5.49^{ab}± 0.78	5.01^{ab}± 1.08	6.43^{b}± 1.28	4.29^{a}± 0.99	4.94^{ab}± 0.91
24th hour	3.92± 0.51	3.59± 0.40	4.26± 0.41	3.64± 0.67	3.87± 0.48
36th hour	3.31^{a}± 0.69	3.74^{ab}± 0.45	3.01^{a}± 0.42	3.62^{ab}± 0.55	4.38^{b}± 0.53
48th hour	5.07^{b}± 1.02	4.22^{ab}± 0.82	4.40^{ab}± 0.67	4.10^{ab}± 0.61	3.77^{a}± 0.62
Reduced glutathione (GSH) (μmol/g protein content)					
12th hour	5.47± 0.76	4.68± 0.75	5.25± 1.13	4.39± 1.21	5.45± 1.03
24th hour	4.39^{a}± 0.79	5.93^{b}± 0.92	6.61^{b}± 0.94	5.74^{b}± 0.53	5.65^{b}± 0.35
36th hour	4.86± 0.82	5.66± 1.44	6.13± 1.11	6.04± 0.99	5.64± 0.44
48th hour	5.72± 0.88	5.41± 0.55	5.46± 0.50	5.52± 0.83	5.18± 0.79
Glutathione peroxidase (GPx) (U/g protein content)					
12th hour	9.35± 3.37	8.59± 3.10	8.67± 3.40	9.77± 2.19	7.75± 1.24
24th hour	9.98^{b}± 1.32	8.25^{a}± 1.21	8.59^{ab}± 0.84	8.53^{ab}± 0.45	8.01^{a}± 0.62
36th hour	8.39^{ab}± 1.64	8.56^{ab}± 2.25	10.50^{b}± 2.58	8.66^{ab}± 0.91	7.16^{a}± 0.72
48th hour	7.76± 1.97	7.36± 1.28	6.75± 1.11	8.16± 1.70	7.06± 1.50

Aflatoxin: 140 μg AFB_1/kg feed; H1: 300 mg Herbamix Basic Premix/kg feed; H2: 600 mg Herbamix Basic Premix/kg feed; H5: 1500 mg Herbamix Basic Premix/kg feed

^{a,b} Means designated with different letters within the same row mean significant difference ($p<0.05$)

Table 39. Individual and combined effect of aflatoxin and different doses of medicinal herb mixture (Herbamix™) on some parameters of glutathione redox system and lipid peroxidation processes of red blood cell hemolysate (mean±SD; n=6)

	CONTROL	AFLATOXIN	H1 + AFLATOXIN	H2 + AFLATOXIN	H5 + AFLATOXIN
Malondialdehyde (MDA) (μmol/L)					
12th hour	7.47± 1.14	7.77± 1.53	7.30± 0.84	7.59± 1.22	6.42± 1.03
24th hour	8.00± 2.03	8.36± 1.65	7.04± 0.36	8.36± 1.94	6.69± 0.96
36th hour	6.88^{a}± 0.86	10.03^{b}± 0.88	6.98^{a}± 1.15	7.88^{a}± 0.46	6.50^{a}± 0.85
48th hour	7.30± 0.88	7.36± 2.06	7.51± 1.17	8.66± 2.15	7.98± 1.62
Reduced glutathione (GSH) (μmol/g protein content)					
12th hour	14.08± 2.47	14.39± 0.84	12.20± 2.63	14.01± 2.01	13.09± 2.10
24th hour	13.65^{b}± 2.28	10.93^{ab}± 2.57	12.00^{ab}± 1.59	9.08^{a}± 0.56	13.24^{b}± 2.04
36th hour	12.76^{a}± 1.92	12.95^{ab}± 1.28	12.34^{a}± 0.98	14.37^{ab}± 2.83	17.34^{b}± 3.75
48th hour	14.80^{b}± 3.34	16.34^{b}± 3.78	12.30^{ab}± 2.74	8.54^{a}± 1.19	9.86^{a}± 1.04
Glutathione peroxidase (GPx) (U/g protein content)					
12th hour	6.90± 0.95	5.86± 1.04	6.22± 1.19	6.78± 0.56	6.75± 1.32
24th hour	6.54± 1.20	5.59± 1.08	6.79± 2.58	5.02± 0.68	6.56± 1.22
36th hour	7.56± 1.24	7.82± 1.69	7.68± 2.81	7.58± 1.27	9.17± 1.11
48th hour	7.73± 1.36	8.62± 2.46	7.37± 1.33	6.96± 1.47	7.25± 0.69

Aflatoxin: 140 μg AFB_1/kg feed; H1: 300 mg Herbamix Basic Premix/kg feed; H2: 600 mg Herbamix Basic Premix/kg feed; H5: 1500 mg Herbamix Basic Premix/kg feed

^{a,b} Means designated with different letters within the same row mean significant difference ($p<0.05$)

5.4.1.2. Parameters of glutathione redox system and lipid peroxidation processes in tissue homogenates

As effect of aflatoxin alone, level of conjugated dienes and conjugated trienes, the initial phase markers of lipid peroxidation processes, did not change significantly in liver, while in combination with herbal mixture significantly lower conjugated triene values were measured at 48h in aflatoxin + medium Herbamix dose group and in aflatoxin + highest Herbamix dose group as compared to control (Table 40).

In liver homogenates, MDA concentration was significantly higher at 12h in aflatoxin treated group as compared to control (Table 41). At 24h of mycotoxin exposure in aflatoxin + medium dose of Herbamix treatment significantly higher MDA concentration was measured as compared to control and to the other herbal mixture treated groups (Table 41).

At 24h sampling, GSH concentration in liver homogenates of aflatoxin treated group significantly exceeded the values measured in aflatoxin + different dose of Herbamix pre-treated groups (Table 41).

Similarly to the changes in its co-substrate availability, the highest GPx activity in liver homogenates were measured at 24h in aflatoxin treated group, which was significantly higher than in control (Table 41).

Table 40. Individual and combined effect of aflatoxin and different doses of medicinal herb mixture (Herbamix™) on some parameters of glutathione redox system and lipid peroxidation processes of liver homogenates (mean±SD; n=6)

	CONTROL	**AFLATOXIN**	**H1 + AFLATOXIN**	**H2 + AFLATOXIN**	**H5 + AFLATOXIN**
Conjugated dienes (CD) (ABS 232 nm)					
12th hour	0.836± 0.050	0.816± 0.058	0.801± 0.069	0.809± 0.047	0.738± 0.079
24th hour	0.706± 0.048	0.775± 0.043	0.748± 0.032	0.743± 0.042	0.763± 0.040
36th hour	0.621± 0.039	0.673± 0.026	0.667± 0.031	0.627± 0.121	0.642± 0.051
48th hour	0.717± 0.024	0.660± 0.041	0.696± 0.049	0.681± 0.025	0.692± 0.033
Conjugated trienes (CT) (ABS 268 nm)					
12th hour	0.246± 0.017	0.233± 0.013	0.230± 0.021	0.242± 0.013	0.235± 0.021
24th hour	0.225± 0.014	0.239± 0.011	0.235± 0.012	0.229± 0.010	0.228± 0.013
36th hour	0.207± 0.008	0.222± 0.011	0.221± 0.013	0.212± 0.027	0.206± 0.012
48th hour	0.230^{b}± 0.007	0.212^{ab}± 0.015	0.220^{ab}± 0.016	0.211^{a}± 0.006	0.211^{a}± 0.010

Aflatoxin: 140 µg AFB_1/kg feed; H1: 300 mg Herbamix Basic Premix/kg feed; H2: 600 mg Herbamix Basic Premix/kg feed; H5: 1500 mg Herbamix Basic Premix/kg feed

^{a,b} Means designated with different letters within the same row mean significant difference ($p<0.05$)

Table 41. Individual and combined effect of aflatoxin and different doses of medicinal herb mixture (Herbamix™) on some parameters of glutathione redox system and lipid peroxidation processes of liver homogenates (mean±SD; n=6)

	CONTROL	AFLATOXIN	H1 + AFLATOXIN	H2 + AFLATOXIN	H5 + AFLATOXIN
Malondialdehyde (MDA) (μmol/g fresh weight)					
12th hour	3.00^{a}± 0.62	5.52^{b}± 1.59	3.58^{ab}± 1.10	4.55^{ab}± 2.26	4.28^{ab}± 0.57
24th hour	3.12^{a}± 0.67	3.83^{ab}± 0.61	3.35^{a}± 0.65	4.82^{b}± 1.06	2.68^{a}± 0.62
36th hour	3.76± 1.73	4.33± 1.85	4.50± 0.73	4.40± 1.38	4.17± 1.3
48th hour	2.81± 0.68	2.72± 0.91	2.74± 0.75	3.08± 0.95	2.88± 1.20
Reduced glutathione (GSH) (μmol/g protein content of 10,000 g supernatant)					
12th hour	5.52^{ab}± 0.51	5.79^{ab}± 0.62	6.59^{b}± 1.02	5.73^{ab}± 0.91	4.78^{a}± 0.66
24th hour	4.65^{ab}± 0.65	5.37^{b}± 0.62	4.13^{a}± 0.33	4.14^{a}± 0.31	4.43^{a}± 0.29
36th hour	4.59± 0.41	5.07± 0.49	5.08± 0.89	4.59± 1.00	5.03± 0.86
48th hour	3.80± 0.35	3.49± 0.49	3.81± 0.78	3.45± 0.26	3.62± 0.40
Glutathione peroxidase (GPx) (U/g protein content of 10,000 g supernatant)					
12th hour	5.23± 0.77	4.99± 0.60	6.11± 1.09	5.54± 0.85	5.30± 0.87
24th hour	4.83^{a}± 0.48	6.14^{b}± 0.71	5.18^{ab}± 0.48	5.18^{ab}± 1.11	5.29^{ab}± 0.42
36th hour	3.97± 0.43	4.23± 0.65	4.41± 0.74	4.03± 0.88	4.20± 0.87
48th hour	3.37± 0.36	3.19± 0.40	3.53± 0.59	3.20± 0.24	3.36± 0.34

Aflatoxin: 140 μg AFB_1/kg feed; H1: 300 mg Herbamix Basic Premix/kg feed; H2: 600 mg Herbamix Basic Premix/kg feed; H5: 1500 mg Herbamix Basic Premix/kg feed

^{a,b} Means designated with different letters within the same row mean significant difference ($p<0.05$)

In kidney homogenates, MDA concentration was significantly higher at 36h in aflatoxin treated group as compared to the control, but 12 hours later it was significantly lower than the control (Table 42). At 48h sampling aflatoxin + medium dose of Herbamix pre-treatment resulted significantly lower MDA concentration in kidney as compared to the control (Table 42).

Although aflatoxin treatment alone resulted elevation in GSH concentration of kidney homogenates at 12h sampling as compared to control, significantly higher values were measured in aflatoxin + medium and highest dose of herbal mixture pre-treatment (Table 42).

GPx activity in kidney homogenates followed the changes of its co-substrate, the highest values were measured at 12h in aflatoxin + medium and highest dose of herbal mixture pre-treated groups (Table 42).

Table 42. Individual and combined effect of aflatoxin and different doses of medicinal herb mixture (Herbamix™) on some parameters of glutathione redox system and lipid peroxidation processes of kidney homogenates (mean±SD; n=6)

	CONTROL	AFLATOXIN	H1 + AFLATOXIN	H2 + AFLATOXIN	H5 + AFLATOXIN
Malondialdehyde (MDA) (μmol/g fresh weight)					
12th hour	2.70± 1.01	5.06± 2.84	4.81± 1.77	4.93± 0.65	4.17± 1.34
24th hour	5.38± 1.72	4.58± 0.77	4.90± 1.19	3.26± 1.90	6.01± 2.98
36th hour	3.14^{a}± 0.79	4.71^{b}± 0.99	2.76^{a}± 0.56	4.18^{ab}± 0.28	3.03^{a}± 1.31
48th hour	3.99^{c}± 0.40	2.65^{ab}± 0.71	3.39^{bc}± 0.50	2.29^{a}± 0.63	3.19^{abc}± 0.37
Reduced glutathione (GSH) (μmol/g protein content of 10,000 g supernatant)					
12th hour	3.98^{a}± 0.28	4.36^{ab}± 0.48	4.77^{abc}± 0.66	5.75^{c}± 1.01	5.22^{bc}± 0.51
24th hour	6.46± 0.75	5.87± 0.78	5.60± 0.49	6.30± 1.23	5.37± 0.76
36th hour	6.24^{b}± 2.08	4.32^{ab}± 1.30	4.03^{ab}± 0.92	6.22^{b}± 2.62	3.23^{a}± 0.54
48th hour	5.66± 3.02	4.67± 1.99	4.56± 0.79	4.97± 1.41	4.33± 0.34
Glutathione peroxidase (GPx) (U/g protein content of 10,000 g supernatant)					
12th hour	3.34**a**± 0.27	3.47**a**± 0.34	3.98**ab**± 0.41	4.86**c**± 0.68	4.45**bc**± 0.72
24th hour	5.64± 0.98	5.31± 1.21	5.31± 0.67	5.61± 0.78	4.41± 0.72
36th hour	5.50± 1.74	3.70± 1.22	3.63± 0.63	5.45± 2.22	3.67± 1.54
48th hour	3.71± 1.17	3.88± 1.12	4.11± 0.54	4.54± 0.60	4.25± 0.31

Aflatoxin: 140 μg AFB_1/kg feed; H1: 300 mg Herbamix Basic Premix/kg feed; H2: 600 mg Herbamix Basic Premix/kg feed; H5: 1500 mg Herbamix Basic Premix/kg feed

^{a,b,c} Means designated with different letters within the same row mean significant difference ($p<0.05$)

5.4.1.3. Clinical biochemical parameters in blood plasma

In case of aflatoxin + medium dose herbal mixture combination aspartate-aminotransferase (AST) activity of blood plasma showed significantly higher values as compared to the control at 48h sampling (Table 43).

Aflatoxin + highest dose of herbal mixture combination resulted significantly higher gamma-glutamyltransferase (GGT) activity than in control at 12h sampling, but 12 hours later it was significantly lower as compared to the aflatoxin treated one (Table 43).

At 12h sampling, glucose concentrations in blood plasma were significantly lower in aflatoxin + medium and highest dose herbal mixture combination as compared to control (Table 43). At 24h sampling glucose concentration in aflatoxin + highest dose of herbal mixture pre-treated group was significantly higher than in the aflatoxin treated group (Table 43).

No significant changes were found in cholesterol concentrations of blood plasma, but triglycerides showed marked elevation in aflatoxin treated group at 36h sampling as compared to control and to aflatoxin + lowest dose of herbal mixture pre-treated group ($p<0.05$) (Table 43).

No significant differences were found in uric acid concentrations of blood plasma (Table 43).

Table 43. Individual and combined effect of aflatoxin and different doses of medicinal herb mixture (Herbamix™) on some clinical biochemical parameters of blood plasma (mean±SD; n=6)

	CONTROL	**AFLATOXIN**	**H1 + AFLATOXIN**	**H2 + AFLATOXIN**	**H5 + AFLATOXIN**
Aspartate aminotransferase (AST) (U/l)					
12th hour	155.90± 29.61	197.00± 32.52	169.50± 17.16	185.90± 18.49	185.80± 28.62
24th hour	172.50± 14.19	169.30± 38.67	173.10± 40.97	159.50± 22.47	172.60± 15.77
36th hour	159.40± 28.13	147.70± 40.77	166.80± 23.53	165.50± 27.33	153.50± 5.21
48th hour	123.00^{a}± 22.44	135.30^{ab}± 14.72	130.90^{ab}± 9.33	149.20^{b}± 9.24	131.20^{ab}± 13.14
Gamma-glutamyltransferase (GGT) (U/l)					
12th hour	36.05^{a}± 8.91	59.04^{ab}± 25.49	56.65^{ab}± 13.19	62.36^{ab}± 23.50	69.83^{b}± 14.83
24th hour	81.25^{ab}± 31.15	116.40^{b}± 77.59	58.29^{ab}± 17.74	52.92^{ab}± 14.42	48.14^{a}± 23.89
36th hour	57.41± 20.54	64.27± 37.70	54.23± 17.21	80.63± 30.97	60.36± 28.63
48th hour	37.42± 16.29	35.30± 13.07	51.89± 27.62	45.10± 16.31	25.88± 6.04
Glucose (mmol/l)					
12th hour	8.95^{c}± 0.47	7.90^{abc}± 0.57	8.45^{bc}± 0.84	7.96^{ab}± 0.10	7.45^{a}± 0.49
24th hour	7.57^{ab}± 0.40	6.83^{a}± 0.53	7.06^{a}± 0.82	7.33^{ab}± 0.68	8.20^{b}± 0.15
36th hour	8.52± 0.42	8.10± 0.65	8.50± 0.92	8.06± 0.62	8.50± 0.74
48th hour	7.29± 0.56	8.15± 0.52	8.01± 0.40	7.89± 0.54	7.69± 0.79
Cholesterol (mmol/l)					
12th hour	3.13± 0.31	3.33± 0.54	2.98± 0.20	2.97± 0.44	2.89± 0.28
24th hour	3.14± 0.24	3.19± 0.36	3.06± 0.64	3.34± 0.38	2.93± 0.36
36th hour	3.57± 0.24	3.73± 0.65	3.64± 0.34	3.50± 0.36	3.53± 0.38
48th hour	3.34± 0.38	3.86± 0.55	3.61± 0.62	3.55± 0.22	3.43± 0.43

	CONTROL	AFLATOXIN	H1 + AFLATOXIN	H2 + AFLATOXIN	H5 + AFLATOXIN
			Triglycerides (mmol/l)		
12th hour	1.43[a]± 0.20	1.75[ab]± 0.33	1.98[b]± 0.05	1.80[ab]± 0.17	1.88[b]± 0.10
24th hour	2.00± 0.15	2.04± 0.17	1.92± 0.15	1.99± 0.13	1.59± 0.63
36th hour	1.63[a]± 0.52	2.18[ab]± 0.27	2.35[b]± 0.19	1.87[ab]± 0.36	2.28[b]± 0.19
48th hour	1.57± 0.82	1.17± 0.58	1.76± 0.66	1.04± 0.42	1.39± 0.38
			Uric acid (µmol/l)		
12th hour	322.60± 49.68	368.10± 107.20	362.50± 33.19	422.10± 90.35	440.20± 97.42
24th hour	442.70± 76.93	547.00± 68.09	523.30± 134.00	489.80± 100.70	436.70± 42.75
36th hour	437.30± 61.83	483.70± 80.96	386.00± 92.53	364.70± 42.97	460.70± 117.80
48th hour	498.10± 120.60	434.60± 54.75	439.70± 113.90	419.60± 98.33	422.00± 147.70

Aflatoxin: 140 µg AFB_1/kg feed; H1: 300 mg Herbamix Basic Premix/kg feed; H2: 600 mg Herbamix Basic Premix/kg feed; H5: 1500 mg Herbamix Basic Premix/kg feed

[a,b,c] Means designated with different letters within the same row mean significant difference ($p<0.05$)

5.4.2. Discussion

The results revealed that short-term AF treatment did not have any effect on feed intake, body, absolute and relative liver weights, which is probably due to the short (48 hours) period of investigation. These results are contrary with long-term feeding of AF contaminated diet, as that resulted marked decrease in feed intake of broilers, and due to fatty infiltrations and enlargement of hepatic cells the liver weight increased (Yunus et al., 2011).

Compared to control, MDA concentration was significantly lower in blood plasma of AF + highest Herbamix dose at 48h, while in RBC haemolysates of AF treated group it was increased at 36h. This result suggests that phytobiotic supplementation reduced the lipid peroxidation in blood plasma, but not in RBC haemolysates, which supports the previous findings in a short-term AF exposure with laying hens (Chapter 5.2.).

GSH concentration in blood plasma increased at 24h in all experimental groups, which suggests response of the organism to oxidative stress as provoked by AF exposure, while in RBC haemolysates the AF + medium Herbamix dose resulted significantly lower concentrations at 24h and 48h compared to control. This is probably caused by the limited *de novo* synthesis of glutathione in RBCs. However, glutathione content of erythrocytes plays an important role in mitigating the damaging effects of reactive oxygen species (ROS) present in the circulation (Mak et al., 1994), which may cause continuous oxidation of hemoglobin within the cytosol of the erythrocyte (Hsieh and Jaffe, 1975). GSH content of erythrocytes possibly decreased because it reacts directly with ROS or act as co-substrate of glutathione peroxidase or glutathione S-transferases (Pace et al., 2003).

GPx activity of blood plasma was significantly lower in AF treated group at 24h as compared to control, while no significant changes were found in RBC haemolysates. This result is similar to the findings of our previous experiment with laying hens (Chapter 5.2.), where GPx activity also decreased in blood plasma, but not in RBC haemolysates. These differences in blood plasma and RBC are possibly caused by the different site of *de novo* synthesis of GPx, because blood plasma GPx is synthetized in the proximal tubular cells of kidney (Avissar et al., 1994).

In case of aflatoxin + medium dose herbal mixture combination aspartate-aminotransferase (AST) activity of blood plasma showed significantly higher values as compared to the control at 48h sampling. Aflatoxin + highest dose of herbal mixture combination resulted significantly higher gamma-glutamyltransferase (GGT) activity than in control at 12h sampling, but 12 hours later it was significantly lower as compared to the aflatoxin treated one.

A meta-analysis based on 98 articles of the relationship of aflatoxin with biochemical parameters in broilers reported significant increase in AST and GGT activities (Andretta et al., 2012). In contrary, another study done by Kumar et al. (1993) reported marked decrease in AST activity in Japanese quail fed 0.5 to 2.0 mg/kg aflatoxin for 30 days. Elevated GGT activity was also reported in broiler chicken fed 0.3 mg/kg aflatoxin and 3 mg/kg T-2 toxin at 35 days of age (Raju and Devegowda, 2000). In Japanese quail chicks Madheswaran et al. (2004) reported significantly higher AST activity, and markedly, but not significantly, higher GGT activity as a result of feeding diets containing 3 mg/kg

aflatoxin for 35 days. The results of present study suggest that herbal mixture at the dose applied moderately, but not completely eliminates the liver damaging effect of aflatoxin.

Glucose concentrations in blood plasma at 12h sampling was significantly lower in aflatoxin + medium and highest dose herbal mixture combination as compared to control. At 24h sampling glucose concentration in aflatoxin + highest dose of herbal mixture pre-treated group was significantly higher than in the aflatoxin treated group. In a meta-analysis Andretta et al. (2012) showed that aflatoxin decreased glucose concentration of blood plasma, but in present study such effect was found in early phase of aflatoxin exposure (at 12h), but phytobiotic treatment eliminated this effect up to 24 h, which suggests its effectiveness against aflatoxin-related impairment of carbohydrate metabolism.

In cholesterol concentrations of blood plasma, no significant changes were found, but triglycerides showed marked elevation in aflatoxin treated group at 36h sampling as compared to control and aflatoxin + lowest dose of herbal mixture pre-treated group. These changes are contradictory with previous studies (Andretta et al., 2012), where significant decrease was found in cholesterol and triglycerides as effect of aflatoxin. The possible cause of these differences can be the short duration of exposure, or possibly the effectiveness of herbal mixture against detrimental effects of aflatoxins on lipid metabolism.

In uric acid concentrations of blood plasma, no significant differences were found. On the other hand, Ghahri et al. (2010) reported reduction in uric acid values in a long-term study with broilers receiving a diet naturally contaminated with aflatoxins (254 μg/kg total AF). In a long-term experiment with Japanese quail done by Madheswaran et al. (2004), high aflatoxin intake (3 mg aflatoxin /kg feed) did not reveal significant difference in serum uric acid concentration. These differences suggest that aflatoxin has dose-dependent effect on uric acid level, but regarding this parameter the effect of herbal mixture was not proven.

According to the results addition of herbal mixture had moderate effect against the mild oxidative stress as caused by aflatoxins at the dose applied, and it also had some effects on glucose and lipid metabolism.

6. CONCLUSIONS AND SUGGESTIONS

6.1. Conclusions

The main purpose of my thesis was to study the effects of dietary herbal mixture supplementation on the effect of aflatoxin B_1, T-2 toxin or DON on the lipid peroxidation processes and the antioxidant defense system. Initial and terminal markers of lipid peroxidation and amount or activity of the glutathione redox system were measured in three-week old broiler chickens and 49-week old laying hens in short-term (48 hours) mycotoxin exposure.

The transit time of feed particles in the gastrointestinal tract, which may influence the time available for the absorption of mycotoxins in the intestine, was also evaluated.

Markers of lipid peroxidation, from initiation phase (conjugated dienes and conjugated trienes) till metastable end-product of terminal phase (malondiadehyde) were measured in liver and kidney. Among the parameters of the biological antioxidant system reduced glutathione content and glutathione peroxidase activity was measured in the same tissues.

It is known that broiler chicken and laying hen are less sensitive to trichothecene mycotoxins than other monogastric farm animals, and their susceptibility is relatively low among the domesticated poultry species. This was supported by the results, that no mortality or morbidity was observed at the applied doses during the short-term trials.

The results revealed AFB_1-initiated lipid peroxidation even in short-term exposure, at the dose applied. This was confirmed by the significant increase of the termination phase parameter (malondialdehyde) in the blood plasma and red blood cell haemolysate, but it was not observed in liver and kidney. These results were in contrast to some previous studies where aflatoxin B_1 exposure resulted in significant increase of malondialdehyde content in the liver. However, those previous studies were based on long-term exposure. The results also showed that aflatoxin B_1 exposure in combination with medicinal herb mixture caused lower level of oxygen free radical formation, but only in layers. Otherwise activation of glutathione redox system was also found, but medicinal herb mixture had only minor effect on it. The most intensive changes were observed about 12 hours after start of feeding mycotoxin contaminated diet, which at first can be explained with the transit time of feed particles and estimated period for absorption in poultry.

The investigated trichothecene mycotoxins, primarily the T-2 toxin and less the DON, also had effect on oxygen radical formation, and subsequently activated the glutathione redox system of broiler

chickens. The addition of herbal mixture had only a moderate effect against the mild oxidative stress generated by T-2 toxin or DON at the dose applied. When the herbal mixture was used as a two-week pre-treatment prior to mycotoxin exposure it had only a moderate effect on the mild oxidative stress as caused by T-2 toxin or DON at the applied dose.

In conclusion, the results indicated that aflatoxin B_1 and the two trichothecenes, T-2 toxin or DON, induced mild oxidative stress either in broiler chicken or laying hen, which was supported by the low level of increase, or even decrease, of the markers of lipid peroxidation, but activation of the antioxidant, namely the glutathione redox system occurred in the same period of time. The results also showed that the phytobiotic supplemetation had some moderate effects, in particular it decreased the rate of lipid peroxidation, without having marked effect on the activation of the glutathione redox system, either if it was used together with mycotoxin exposure or as pre-treatment.

6.2. Suggestions

Based on the individual effect of aflatoxin and trichothecenes it can be recommended to evaluate the effects of multiple mycotoxin exposure, as these mycotoxins are occurring in a mixture in the animal feed and those common effects might be additive, synergistic or antagonistic, which would be important information, when we try to find the most effective method to decrease their detrimental effects on animal production and health.

Additionally, the results of my thesis showed that elimination or attenuation of the negative effects of mycotoxins requires prevention or reduction of the oxidative stress as caused by mycotoxins via action of natural antioxidants or antioxidant mixtures, such a mixture of essential oils or various extracts of medicinal plants. Thereby, these results would be provide more information about the mode of action and efficacy of other natural active substances, supporting the defense against the undesirable effects of mycotoxins in farm animals.

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7. NEW SCIENTIFIC RESULTS

1. The investigated ‘type A’ trichothecene mycotoxin, the T-2 toxin has short-term effect on oxygen free radical formation, and consequently, it activates the glutathione redox system, namely synthesis of reduced glutathione and activation of glutathione peroxidase in liver and kidney of broiler chicken.

2. Addition of herbal mixture, either using together with T-2 toxin, or after two-week pre-treatment prior to mycotoxin exposure, has only moderate effect against the mild oxidative stress caused by T-2 toxin at the dose applied.

3. Short-term exposure of aflatoxin B_1 induces lipid peroxidation, which was confirmed by the significantly increased malondialdehyde content in blood plasma and red blood cell haemolysate, liver and kidney of broilers. These results suggest rapid activation of the antioxidant, namely glutathione redox system in tissues important for the detoxification of mycotoxins.

4. The pre-treatment of medicinal herbal mixture (Herbamix^{TM}) prevented the pro-oxidant effect of aflatoxins, which means lowering the rate of lipid peroxidation and production of malondialdehyde in red blood cells and kidney of broilers.

8. SUMMARY

In temperate climatic conditions cereal grains, which play an important role in the nutrition of monogastric animals, are frequently infected with toxigenic field molds, such as *Fusarium sp.*, producing trichothecene mycotoxins, like deoxynivalenol (DON) and T-2 toxin. In (sub)tropical regions of the world the most important mycotoxins, such as aflatoxin B_1 (AFB_1), are produced by different species of *Aspergillus* genus.

A wide range of the long-term toxic and pro-oxidant effects of the above-mentioned mycotoxins on different poultry species are well known, but there is rare information about their short-term effects.

One of the aims of the series of experiments during my doctoral research was to evaluate the short-term effects of sub-lethal doses of trichothecene mycotoxins (DON or T-2 toxin) or aflatoxin on the lipid peroxidation processes in broiler chickens or laying hens, and on the amount and activity of the glutathione redox system, an important part of the biological antioxidant defense.

Phytobiotics represent a wide range of bioactive compounds derived from different parts of herbs along with plant extracts or essential oils obtained from the plants, embedded into diets to improve livestock productivity. Only a limited number and sometimes contradictory studies are available about the action of phytobiotic supplementation on the antioxidant defense-abilities of chickens against the pro-oxidant effects as caused by mycotoxins.

Therefore, the other purpose of my doctoral work was to evaluate the effects of a commercially available phytobiotic feed additive (Herbamix Basic Premix™) - containing essential oils from 7 plants and glycerol extract of two plants - at different concentrations and period against the detrimental effects of high mycotoxin exposures.

I designed four short-term (48 hours long) studies, one with laying hens and three with broiler chickens.

In my first experiment the effects of herbal mixture and short-term DON (16.12 mg/kg feed) or T-2/HT-2 toxin exposure (3.72 mg/kg feed and 1.26 mg/kg feed, respectively) in 3-weeks-old broiler chickens was investigated, while my second experiment was done with laying hens fed with aflatoxin contaminated diet (125 µg AFB_1/kg).

The trichothecene mycotoxins, at the applied dose, had moderate effect on oxygen free radical formation, and subsequently it caused activation the glutathione redox system of broiler chickens. The

addition of herbal mixture caused only moderate effect against the mild oxidative stress as generated by DON or T-2 toxin at the dose applied.

AFB_1 initiated lipid peroxidation even in short-term, at the dose applied, was confirmed by the significant increase in the termination phase parameter (MDA) in blood plasma and red blood cell haemolysate, but such changes did not appear in the liver and kidney. AFB_1 exposure in combination with medicinal herb mixture caused low oxygen free radical formation. AFB_1-related mild oxidative stress activated the amount/activity of the glutathione redox system, but medicinal herb mixture had only minor effect on it.

The third and fourth experiments aimed to investigate the effects of two-week long herbal mixture pre-treatment and short-term T-2 toxin (1.13 mg/kg feed) or aflatoxin exposure (140 µg AFB_1/kg) in 3-weeks-old broiler chickens.

T-2 toxin or aflatoxin have induced mild oxidative stress in broiler chickens, which was supported by the low level of increase, or even decrease of the markers of lipid peroxidation, resulted in activation of the glutathione redox system in the same period of time.

The results also showed that the phytobiotic supplementation had some moderate effect, in particular decreasing the rate of lipid peroxidation, without having marked effect on the activation of the glutathione redox system when it was used as pre-treatment.

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