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The impact of the herbal antioxidant AV3 - feed additive on the rabbit metabolism and health status

Master Thesis

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Declaration

I hereby declare that this thesis entitled “The impact of the herbal antioxidant AV3 - feed additive on the rabbit metabolism and health status” is a presentation of my original research work. All the sources have been quoted and acknowledged by means of complete references.

[Kevin Damianus Sutanto]

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Abstract

Since the risks of antibiotics and chemical additives, the trend to use herbal extracts as feed additives has been increasing in this decade. Antioxidant is very important to fight against oxidative stress and the concentration always needs to be maintained. Antioxidant AV3 was comprised of active substances milk thisle (*Silybum marianum*) and ginkgo biloba extracts which are very rich in antioxidant content. The increase demand for rabbit meats drive the utilization of herbal additive to improve both the quality and productivity. In this study, AV3 (0.20 ml/kg) was administered in healthy fattening rabbits (n=90) to evaluate the changes of blood biochemistry (ALT, AST, ALP, total protein, albumin, globulin, amylase, cholesterol, urea, phosphorus, calcium and glucose), reduced antioxidant (GSH) and growth parameters (slaughter weight, daily feed intake, daily gain, feed conversion, total weight increase, total feed consumption, liver weight, kidney weight, carcass weight, carcass yield). The mortality percentage was also counted. The supplementation of AV3 made no significant changes in most of blood biochemistry except for cholesterol. Cholesterol level was elevated significantly by 38.6% compared to the control group. Similarly, the addition of AV3 did not make considerable impact on GSH and most of growth parameters. GSH level was insignificantly enhanced in the AV3 group. The mortality rate was not positively influenced by AV3 treatment. AV3 treatment caused carcass yield and kidney weight changed significantly from 57.22% to 58.31% and by 8.1% respectively. The inclusion of 0.20 ml/kg AV3 had little to no importance for fattening rabbits in terms of the overall health and growth performances. Even though there were no negative effects, there is a need to consider the value from the economical perspective.

Keywords : rabbits, flavonoids, silymarin, ginkgo biloba, blood, antioxidant

Abstrakt

Z důvodu rizik spojených s antibiotiky a chemickými přídavky se v posledním desetiletí zvedá zájem o použití rostlinných extraktů jako krmných doplňků. Antioxidanty jsou velmi důležité k potlačení oxidačního stresu a jejich koncentrace se musí udržet. Antioxidant AV3 sestává z aktivní substance ostrořepce mariánského (*Silybum marianum*) a ginkgo biloby, které jsou oba velmi bohatí na obsah antioxidantů. Zvýšená poptávka po králíčím masu vede k použití rostlinných příměsí na zvýšení jak kvality, tak i produktivity. V této práci, AV3 (0,20 ml/kg) byl nasazen na výkrm králíků (n = 90) na vyhodnocení změn ve složení krve (ALT, AST, ALP, celkový protein, albumin, globulin, amylase, cholesterol, urea, fosfor, kalcium a glukóza), redukované antioxidanty (GSH) a růstové parametry (porážková hmotnost, denní krmná dávka, denní přírůstek, přeměna krmiva, celkový hmotnostní nárůst, celková spotřeba krmiva, váha jater, váha ledvin, váha mrtvoly, výnos mrtvoly). Procento úmrtí také bylo započítáno. Suplementace AV3 neměla žádný zvláštní vliv na biochemii krve krom cholesterolu. Cholesterol stoupl významně o 38,6 % v porovnání s kontrolní skupinou. Podobně, přídavek AV3 neměl významný dopad na GSH a většinu růstových parametrů. Úroveň GSH se významně zvedla ve skupině s AV3. Úmrtnost se pozitivně nezvýšila díky AV3. AV3 vvýrazně změnil výnos mrtvoly i hmotnost ledvin z 57,22% na 58.31% a změna o 8,1%. Zahrnutí 0,20 ml/kg AV3 mělo malý až žádný vliv na výkrm králiků v ohledu na jejich zdraví nebo růst. I když tu nejsou žádné negativní efekty, není zahrnutí AV3 nezbytně potřebné z ekonomického hlediska.

Klíčová slova: králíci, flavonoidy, silymarin, ginkgo biloba, krev, antioxidanty

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1. Introduction

Rabbit meat is a lean meat, it has low cholesterol, fat, and saturated fatty acid content. The high nutrition and meat properties of rabbit meat makes it is valued and in demand especially in some countries. For some countries, especially, in Europe and North Africa, rabbit meat also contributes to generating income and thus the development of the countries. According to FAO, the demand for rabbit meat increase up to 1.68 tonnes in 2010. Main producer countries in Europe are Italy, Spain, France, Czech Republic and Germany (Nistor et al., 2013; Hernandez, 2008).

People nowadays tend to take more nutritive value and safety of meat products into account (Hernandez, 2008). The nutritional contents in the meat of rabbits can be easily enriched through feed consumption. Many growth promoters have been used to increase the production capacity. However, the regulation of European Union in 2003 prohibited the use of antibiotics as well as chemotherapeutic drugs due to possible subsequent health concern (Khan et al., 2012). Lipid oxidation is of great importance in rabbit meat since the meat contains high amount of polyunsaturated fatty acids. This makes the meat is vulnerable to oxidation, resulting in colour changes and rancidity (Knekt et al., 1996). Utilization of synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), have been widely used as antioxidants in the food industry, however, it may be responsible for liver damage and carcinogenesis. These conditions inevitably draw people to searching for other safer feed additive alternatives. One solution to this problem is to supplement the diet with medicinal plant and herb sources (Dalle Zotte and Szendrő, 2011; Khan et al., 2012).

A high number of plant extracts have been utilized for human nutrition and health. They are of great importance because of so many potential benefits they have such as antiviral, anti-inflammatory, antimicrobial, antioxidant and other effects. For this reason, they are deemed to be a great alternative to antibiotics to improve performance of animals. They act through several mechanisms such as alteration gut microflora, improving immunity, enzyme stimulation, appetite improvement and suppression of the cultivation of pathogen (Liu, 2012). The outcome of supplementing herb or plant extracts are varied depending on the bioactive contents (Liu, 2012; Demir et al., 2005).

Milk thistle and ginkgo biloba extracts are nowadays frequently researched, especially for medical purposes. Milk thistle extracts are popular for its benefit of being a therapeutic and hepatoprotective agent. While ginkgo biloba has been already used for

medical purposes for thousands of years and now become the most actively researched medicinal plants on earth. G. Biloba is nowadays mainly used to help people with aging related diseases. Both of them possess great antioxidant properties and have been proven to be able to reduce cholesterol level (Blecharz-Klin et al., 2009; Mulrow et al., 2000).

There are many research on implementing milk thistle extract and ginkgo biloba separately, but only a few which are supplementing them together, especially in rabbits. In this research, milk thistle and ginkgo biloba extracts will be added simultaneously in order to obtain a synergistic result. Blood biochemical properties are very important to measure the overall health and nutritive value of animals.

2 Literary review

2.1 Reactive oxygen species

Free radicals have been a term of highly reactive molecules with unpaired electrons in their atomic orbitals. Reactive oxygen species (ROS) are greatly reactive molecules, atoms or ions containing oxygen, including free radicals (Lobo et al., 2010). They are very unstable and keep trying to capture electrons to obtain stability, causing damage to surrounding molecules. Several deleterious damages caused by ROS are membrane lipid peroxidation due to oxidation, cell and DNA damages, lower membrane fluidity and DNA mutation.

ROS are synthesized in vivo by organisms in their normal metabolic processes and there are about 1-3% oxygen consumed change into ROS (Coulston and Boushey, 2008). ROS are actually useful in physiological cell processes only if at low to moderate concentrations. However, they can be harmful for lipids, proteins, and DNA in high concentration because of unfavourable alteration of the cell components (Valko et al., 2007). Most of ROS are produced as the outgrowth during mitochondrial electron transport and as metal catalysed oxidation (Turrens, 2003). In that respect there are three primary ROS that are of significant influence in physiology, i.e. superoxide anion (O_2^-), hydroxyl radical (•OH), and hydrogen peroxide (H_2O_2) (Claudio and Niles, 2012).

The formation of superoxide anion is from the addition of one electron into the molecular oxygen (Miller, 1990). Most of the superoxide anion is formed in the mitochondria and NADPH oxidase and mitochondrial electron transport system mediate the process (Sahiner and Cansin, 2012). ROS (Superoxide) are also generated by cells as a result of oxidative burst from phagocytes, that is a part of immune systems by which protect against bacteria, viruses and foreign proteins (Percival, 1998).

O_2^- has a capability to react with hydrogen peroxide and create the most reactive ROS that is OH^- as the product. This hydroxyl can obtain an electron from polyunsaturated fatty acids, thus initiating the lipid peroxidation. Lipid peroxidation occurs as the result of an interaction between unsaturated fatty acids and free radicals in lipid. (Morel, 1983). ROS does not only trigger lipid peroxidation but also disturb the lipid membranes (Girotti, 1985).

Apart from endogenous sources, exogenous sources may also induce ROS to be made. Cells need to detoxify exogenous sources like drugs, UV light, ionizing radiation, numerous kinds of pollution and contaminants (Tedesco et al. 2000), chronic inflammation

so that increase the oxidizing capacity in the body. This natural process is called as xenobiotic metabolism. Exogenous sources induce wound in some regions of mammalian cells, namely erythrocytes, epithelial cells, neutrophils, endothelial cells and other blood elements (Jaimes et al., 2001). Besides the content of free radicals, ROS also contain non-radical forms, which are created when two unpaired electrons are shared. ROS can form as a singlet oxygen, superoxide anion radical, hypochlorite radical, nitric oxide radical, hydrogen peroxide, and various lipid peroxides (Percival, 1998).

In order for cells to halt the deleterious effects of ROS, aerobic organisms have incorporated antioxidant systems. Luckily, antioxidant control ROS they especially stabilize or deactivate free radicals before attacking cells. Hence, antioxidants are highly essential for preserving cellular condition and health for overall (Sinatra and DeMarco, 1995). However, the antioxidant systems can be overpowered if lots of sources induce ROS to produce. Free radical reactions are immediately formed after the first reaction, and they will be accumulated in the body system. This makes antioxidants need to always be available in an adequate amount to overcome them to be debilitating (Sahiner and Cansin 2012).

2.2 Antioxidants

Oxidative stress may occur when cells cannot protect themselves against ROS due to the lack of defence mechanisms, for example under stress condition. In human, the lack of antioxidants attributes to a high number of diseases, including cancer, neurological disorder, hypertension, asthma, inflammatory disease, acute respiratory, and pulmonary disease (Krishnaiah et al., 2011). Antioxidants play a great role in detoxification of ROS, which is therefore crucial for the survival of aerobic organisms. Antioxidants act to achieve the need of protections as well as help to reach balance between ROS creation and removal.

Oxygen homeostasis is regulated by antioxidant through redox reaction involving two compounds, electron donor (oxidized) and receiver (reduced) (Seifried et al., 2007). There are various varieties of antioxidants in the body to counteract the number of dangerous effects of ROS.

Antioxidants are divided into two groups which are enzymatic antioxidants and non-enzymatic antioxidants. Enzymatic antioxidants include glutathione transferase (GST), superoxide dismutase (SOD), catalase (CAT), peroxiredoxin (PRX), thioredoxin (TRX), and glutathione peroxidase (GPx) (Nordberg and Arnér, 2001). Superoxide dismutase

works together with catalase to neutralize destructive effects of ROS. In the beginning, superoxide dismutase help in the reaction of modifying two superoxide anions into one molecule of hydrogen peroxide as a catalyst (Eq.1) through the Haber–Weiss reaction (McCord and Fridovich, 1968; Kehrer, 2000). Catalase will then complete the detoxification process by converting hydrogen peroxide to oxygen and water in the peroxisomes (Eq. 2).

Both reactions are described below (Held, 2010):

Eq. 1 : $2\ O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

Eq. 2 : $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

Fenton reaction may occur when hydrogen peroxide interacts with metal like iron resulting in OH• : $Fe2^+ + H2O2 \rightarrow Fe3^+ + OH\bullet + OH^-$ (Chemizmu and Fentona, 2009).

Whereas non-antioxidant enzymes include :

1. Vitamin and derivatives: Vitamin C (ascorbic acid), vitamin E (tocopherols and tocotrienols), Vitamin A (Retinol).
2. Cofactor : Coenzyme Q10
3. Minerals : Zinc, Selenium
4. Organosulfur compounds: Glutathione
5. Nitrogen non-protein compounds : Uric acid
6. Metal binding proteins: albumin, lactoferin, and ceruloplasmin.
7. Phytonutrients: flavonoids (flavonols, favones), carotenoids (lycopene, lutein,) isoflavones, and terpenoids.

(Kalam et al., 2012; Carocho and Ferreira, 2013)

Vitamin E is lipid-soluble while Vitamin C is water-soluble, both of them possess a identical function in protecting membranes against ROS. Glutathione is one of the preeminent intracellular antioxidants against the damaging effects of ROS. Aside from antioxidant, glutathione also plays roles in the synthesis of DNA and protein, gene expression, cytokine production, immune response, and other in the cellular regulations (Wu et al, 2004)

The overall process of the creation of ROS and how some antioxidants deal with it inside body system is pictured in the diagram below.

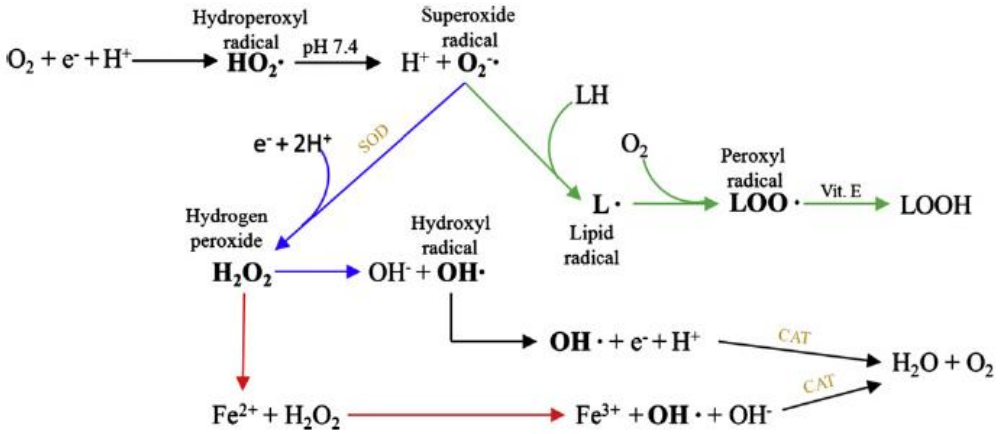

Figure 1. ROS construction overview. The Haber-Weis reaction is represented by the blue arrows. Green arrows display lipid peroxidation while the red arrows represent the Fenton reaction (Carocho and Ferreira, 2013).

In 2013, Ebeid et al. conducted a research using vitamin E and Se to fortify the feed. Vitamin E and Se are known to be excellent antioxidant sources and they have a synergistic relationship to defend cells. Se involves in activating antioxidative properties by means of connecting itself to the active site of the enzyme glutathione peroxidase (GSH-Px) in liver, edible tissues and blood whereas vitamin E is an integral element in lipid membranes. The results indicated that the growth performances had improved when fed by vit. E and organic Se. The explanatory reason was because those natural antioxidants might defend intestinal mucosa against oxidative damage, improve immune systems, and limit peristaltic movements so that they can better digest feed and lessen suffering from diarrhoea. (Ebeit, 2013)

2.2.1 Antioxidant glutathione

In liver, glutathione and its related enzymes (glutathione peroxidase, glutathione reductase and glutathione-S-transferase) are extremely important mainly as protector. Besides, its capability of combating free radicals and then boost immune system, glutathione can also detoxify toxic substances (Czeczot et al., 2006). Glutathione holds three amino acids (glycine, glutamyl and cysteinyl) united by peptide bonds as a tripeptide. Cysteinyl has a sulfhydryl (thiol) side chain as nucleophile which is responsible for donating electrons to electrophilic substances (e.g. ROS and free radical) . Glutathione can be synthesized from glutamic acid, cysteine and glycine so it is categorized as nonessential nutrient. The creation of GSH is mostly present in cytosol and involves two cytosolic enzymes, γ-glutamylcysteine synthetase and GSH synthetase (Lu, 2009)

Glutathione can be present as reduced (GSH) or oxidized (GSSG) forms. During donating electrons to unstable molecules, GSH will become reactive due to electron losses. This condition makes two of reactive GSH joined by a disulfide bridge and glutathione disulfide/oxidized glutathione (GSSG) is formed as the result. It happens because of the high level of GSH in cells (Nordberg and Arnér, 2001). The structure of reduced glutathione and oxidized glutathione are depicted in Figure 2.

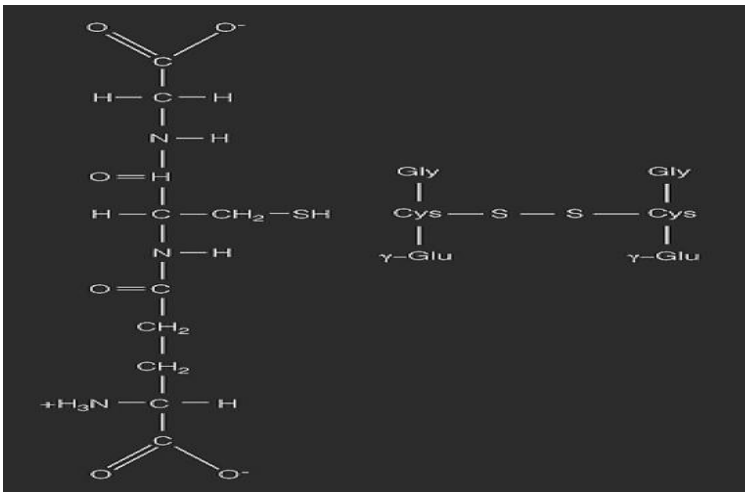

Figure 2. The structure of GSH (left) and GSSG (right) (Stryer, 1988)

2.2.1.1 Biosynthesis of glutathione

Glutathione can be produced in cells throughout the body system, however, glutathione in the liver is the most crucial (Chen et al., 2007). An exceptional GSH amount is produced in the bile of liver containing up to 10 mmol/l GSH, while the concentration in plasma is only 2-20 µmol/l (Jones, 2001 ; Griffith, 1999).

Biosynthesis of glutathione takes place in two adjoining cells, involves enzymatic reactions with the use of ATP and attract amino acids as being substrates. Biosynthesis of glutathione metabolism by means of the γ-glutamyl cycle is globally depicted in Figure 3.

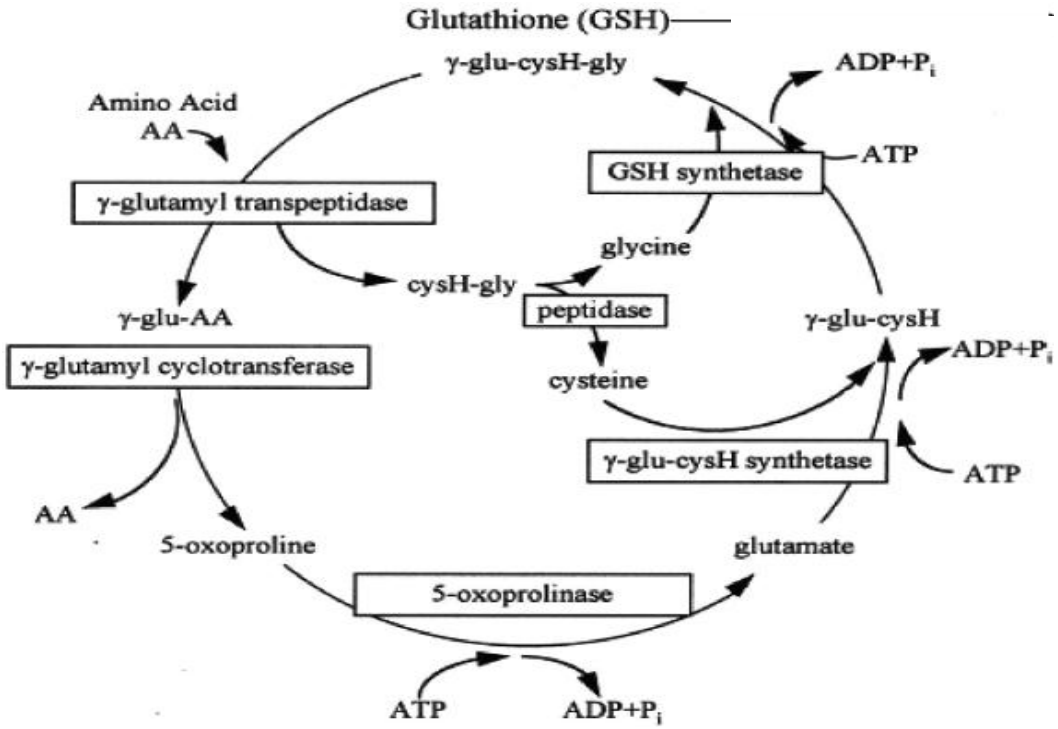

Figure 3. Glutathione synthesis in animal cells (Singh et al., 2013).

In the initial step, L-glutamate and cysteine synthesis γ-glutamylcysteine with the use of γ-glutamylcysteine synthetase. This is a limiting step in GSH synthesis because cysteine is relatively rare to be available in foodstuff (White et al, 2003). In addition, providing decent protein nutrition is essential for maintaining the GSH homeostasis. It is mainly because cysteine is not only produced from methione, but also from the degradation of protein in the diet. Thus, fasting, protein malnutrition or amino acid deficiencies contribute to the limitation of GSH synthesis. In the second step, enzyme GSH synthetase makes glycine to be attached to the C terminal of γ-glutamylcysteine. γ-glutamylcysteine can accumulate if there is no conversion to GSH. This accumulation can lead to the construction of 5-oxoproline by the enzyme γ-glutamylcyclotransferase which may cause metabolic acidosis (Kidd, 1997).

Free radicals and other reactive oxygen species can be directly scavenged by GSH or indirectly using an enzymatic reaction (Fang et al., 2002). In the process of neutralizing oxidant substances, the concentration of GSH is significantly reduced and GSSG is higher in response to oxidative stress, protein/amino acid malnutrition, and other pathological states (Brigelius et al., 2002). Nevertheless, enzyme glutathione disulfide reductase catalyses the GSH recycle from GSSG by utilizing NADPH as the electron donor in a reversible reaction (Couto et al, 2013). The glutathione reductase reaction is described below (Cohen and Hochstein, 1963) :

$$GSSG + NADPH + H^{+} \xrightarrow{\text{GSH reductase}} NADP^{+} + 2GSH$$

The total concentration of glutathione in cells can be indicated from GSH + 2GSSG in which some of them, less than 15%, is bounded to protein (Wu et al., 2004). The ratio of [GSH] to [GSSG] is commonly used as an indicator or marker for oxidative stress and to determine the antioxidative capacity of cells. Under normal conditions, the ratio of [GSH] to [GSSG] is 10:1 and ratio may be attributed by several other redox couples (eg. NADPH/NADP+) (Jones, 2001; Griffith, 1999). The mechanism reaction of GSH in reducing H_2O_2 molecules is shown below (Cohen and Hochstein, 1963) :

$$H_2O_2 + 2GSH \xrightarrow{\text{GSH peroxidase}} 2H2O + GSSG$$

$$ROOH + 2GSH \xrightarrow{\text{GSH peroxidase}} 2ROH + GSSG$$

2.2.2 Antioxidant in blood

There are various reasons of making red blood cells (erythrocytes) to be a standard oxidative stress examination. First, erythrocytes are exposed to high oxygen level as they carry the oxygen and therefore can also be deemed as circulating antioxidant carrier. Second, they cannot mend or change the broken components. Third, polyunsaturated fatty acid chains which can be found as a composer of membrane lipids are highly susceptible to peroxidation. Lastly, antioxidant enzymes are available in red blod cells (Konyaliogle and Karamenderes, 2005).

In red blood cells, the main element that cause deleterious effects come from hydrogen peroxide. H_2O_2 will react with haemoglobin produced in erythrocytes and convert it into its oxidized forms (methaemoglobin and ferrihaemoglobin), which are greatly involved in oxidative processes (Tedesco et al, 2000).

Antioxidant enzymes may be sufficient in giving protection against ROS only if in under normal conditions. However, exogenous antioxidants need to be supplied in order to compensate the ROS productions when there are unavoidable toxic substances from environments or under pathological states (Kiruthiga et al, 2007).

2.3 The use of plant secondary metabolites as feed additives.

Plants synthesize a huge number of organic materials which are divided into two categories, primary and secondary metabolites. Primary metabolites are organic materials

produced by the plant that have direct functions in growth and metabolism including solute transport, translocation, nutrient assimilation, respiration and photoshyntesis (Eg. Nucleotides, amino acids, phytosterol, and organic acids) (Crozier et al., 2008). While secondary metabolites, on the other hand, are bioactive substances synthesized by plants that are not involved in 'primary' metabolic pathway. Secondary metabolites play great roles, especially in environmental adaptation. Secondary metabolites are very important to give protection against UV, microorganism (Eg. Viruses, bacteria and fungi, as they have antiviral, antibacterial and antifungal functions) and herbivores (arthropods and vertebrates) (Wink, 1988; Mazid et al., 2011). The more secondary metabolites are synthesized, the more endurance will the plants have. Besides giving plant protection, the other functions are allelopathic agent, signalling molecules, and pollinators. It also contributes to odours, tastes and colours of plants, making each plant different among others (Pichersky and Gang, 2000; Bennett and Wallsgrove, 1994).

Secondary metabolites bring enormous benefits for human and have been used as traditional medicines for hundreds of years. These days, people are very dependent on them, since a high number of products such as cosmetics, drugs (antibiotics), chemical (dyes, insecticides), and so on, contain secondary metabolites. Since those benefits, numerous researches to produce plant secondary metabolites such have been done (Rao and Ravishankar, 2002). As the population of the world grows, the need of plant derived product also increase, the US market demand for plant based medicines increase amount to approximately US$ 3 billion per year (Glaser, 1999). There approximately 75% people on earth have relied on plant substances for traditional medications. In the US and Europe, pharmaceutical industry utilizes plant based materials of up to 25% (Bourgaud et al., 2001; Rao and Ravishankar, 2002).

The use of secondary metabolites is also very potential for animal nutrition. They are deemed to be the bioactive compounds in phytogenic feed additives (Costa et al., 2013). Feed additives are animal feed supplements given by farmers that improve the dietary feed in order to get the desired responses (better performances and health) (Hashemi and Davoodi, 2010). So, the usage is completely different from veterinary drugs which are used only for prophylaxis and therapy in the short period of time.

While phytogenic feed additives are feed additives obtained from plant sources, Eg. Herbs, spices, essential oils, or oleoresins (Windisch et al., 2008). The examples of feed

additives which are commonly used are performance promoters, antioxidants, emulsifiers, stabilizers, flavours, and probiotics (Wenk, 2000).

The application of plant secondary metabolites as feed additives, however, is still recent compared to human-related usage so there are still relatively limited results of the trials (Nielsen,2008). The trend is becoming apparent, especially in Europe after the regulation of the European Union to ban the utilization of antibiotics as growth promoters caused by the growing concern for the risk of health that may impose (Chesson, 2004). In 2002, a German annual feed additive related conference "Society of Nutrition Physiology" stated nearly half of papers on feed additives connected with herbs to improve performances and carcass characteristics (Weng, 2003). The demand for organic animal products have been also increasing, considering the perception that organically farmed animals are healthier. For these reasons, considerable attention to the benefit of natural additives like secondary metabolites in animal has been given (Nielsen,2008; Wallace,2004).

A great diversity of secondary metabolites which is produced by plants not always give positive impact on animal health (Wallace,2004). There are some compounds which are harmful to animals, for example linamarin in cassava (Cereda and Mattos, 1996). A large number of results showed promise of the efficacy of plant secondary compounds. Some secondary metabolites have a positive effect in intestinal digestions influencing positively nutrient digestibility while some others affect on tissue metabolism, change in colour, flavours, immunity, egg quality, and many others (Mareš et al., 2008; Wenk,2000). Most of secondary metabolites benefit to animals belong to flavonoids, isoprene, and glucosinolates (Wenk, 2003). Nielsen claimed the use of citrus, chestnut, grape, green tea extract, chestnut wood and white willow bar and oil as feed additives in piglets and finishers could improve daily gain and feed utilization (Nielsen, 2008). In addition, Tanins and saponins in the pods of *Acacia petanula* and *Enterolobium cyclocarpum* can assist in alleviating methane in rumen (Acton, 2013).

2.3.1 Flavonoids

Plant secondary metabolites are classified into three main categories according to their biosyntheses, namely flavonoids, terpenoids and nitrogen-containing alkaloids and sulphur-containing compounds (Agostini-Costa et al., 2012). There are more than 8000 phenolic compounds or poliphenols have been identified. They are major constituents of

constructing plant secondary metabolites and among other molecules, flavonoids are the most prevalent phenolic compounds (Bravo, 1998). These phenolic compounds are effective to be antibacterial, antiallergic, hepatoprotective antiviral, anticarcinogenic, antithrombotic and anti-inflammatory agents (Middleton et al., 2000). Most of their effectiveness is related to their antioxidant and radical scavenging. (Soobrattee et al., 2005).

Flavonoids have structure of C6-C3-C6 carbon skeleton and they may exist with sugar group (glycosides) or without sugar group (aglycones) (Peterson and Dwyer, 1998).

There are six classifications of flavonoids, include flavones (tangeritin and luteonin), flavonols (kaemferol and quercetin), flavanones (naringenin and eriodictyol), isoflavones (glycitein and genistein), flavans (catechins and epicatechins), and anthocyanins (cyanidin and petunidin) (Ghasemzadeh and Ghasemzadeh, 2011).

Flavonoids are comprised of several catergories in respect to structural class (heterocyclic ring), molecular size and other substitution (Eg. isoprenylation) (Shahidi, 1997; Hollman, 1997). Flavonoids can be also located in immune system, cell systems, and homeostasis of animals. Many effects of flavonoids on mammalian enzyme systems were reported previously which many of them take part in cell division, proliferation, immune responses, detoxification and inflammatory (Middleton and Kandaswami, 1994).

2.3.1.1 Flavonoid as antioxidant

Strong antioxidant activities possessed in flavonoids has led to gain more interest in the recent research due to their potential health benefits. They may either be able to scavenge free radicals owing to their functional hydroxyl groups or to chelate metals ions (Kumar and Pandey, 2013). The scavenging free radicals activity of flavonoid may inhibit the process of lipid peroxidation and oxidation of LDL (Grotewold, 2006). Lipid peroxidation is a natural process of oxidation that involved lipid on which free radicals attack, especially on PUFA (polyunsaturated fatty acids) region due to the high number of carbon-carbon double bonds. (Ayala et al., 2014). In addition, phagocytes activities can also trigger the reaction to occur. The outcome of this reaction can be dangerous for the viability of both cells and tissues that may lead to atherosclerosis and cancer. Self defence mechanisms such as enzymes, vitamin and natural antioxidants exist, however they can be overcome. (Mylonas and Kouretas, 1998). Basically, flavonoids block the initial step of 3-step reaction process of lipid peroxidation by performing as scavengers. Nevertheless,

there are some flavonoids act as metal-chelating agents that hinder superoxide driven Fenton reaction (Cook and Samman, 1996).

Milk thistle and Gingo Biloba are herbs that currently commonly used as herbal medicines or supplements as well as feed additives due to their high flavonoid content serving as antioxidant.

2.4 Milk thistle (*Silybum marianum*)

There are many synthetic antioxidants accessible, however, natural products (flavonoids, carotenes, polyphenol, vitamins, andlycopeness) are more in demand. The growing trend towards natural products drive the use of phytochemical in several herbs and spices for examples rosemary, turmeric, white pepper, ginger, and medicinal plants (Nakatani, 1997; Jitoe et al., 1992)

Fig.4. Milk thisle

www.manghebati.com

Milk thistle (*Silybum marianum*) is a plant originally from the Mediterranean and North African regions, but already it wildly grows across Europe, America and Australia (Boulos, 2000; Hamid et al., 1983). It can grow up to 10 feet in height and has white patches along the veins of the leaves that may distinguish from other plants. Milk thistle has been commonly used as a medicine for over 2000 years, especially for liver disorders (Kroll et al., 2007; Post-White et al., 2007).

Milk thistle contains a crude extract bioflavonoids silymarin, which can be separated from the seeds and fruits of the milk thistle. Silymarin represents 65-80% of milk thistle extracts and consists of more than 7 flavonolignans and one flavonoids, a taxifolin (Davis-Searles et al., 2005; Kroll et al., 2007). Silibyn, silychristin and silyadin are some examples of flavonolignans that constitute of silymarin. Among them, silibyn is the most available flavonolignans and is divided into diastereoisomers silybin A and silybin B to ratio 1:1. There are also present the regiomers of silybin A and silybin B so called as diastereoisomers isosilybin A and isosilybin B with also have a ratio 1:1 (Figure 5).

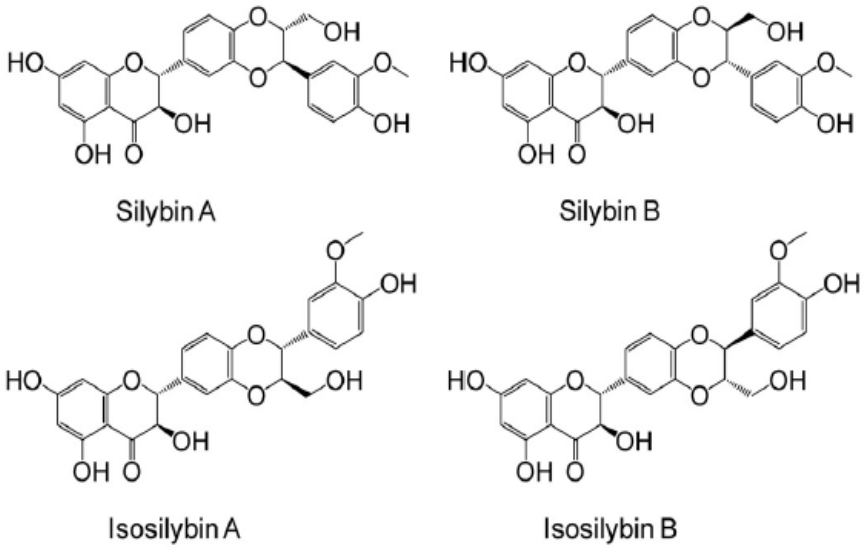

Figure 5. Structure of diasteriomers silibyn and isosilybin (Davis-Searles et al., 2005).

2.4.1. Silymarin as antioxidants

These days, silymarin and silibyn extracts have been used for the treatment of numerous diseases (e.g. Hepatitis, cirrhosis, alcohol-related diseases, and noxious exposure) (Nijveldt et al., 2001).

Organic extract compounds from plants usually have the capability of scavenging ROS and of providing endogenous defence structures to combat oxidative stress. Researches have been conducted on silymarin as a functional antioxidant. It can act as an O_2^- scavenger, immunostimulator, glutathione content enhancer, and it can also recover cellular antioxidant protections (Alidoost et al., 2006).

The scavenging of free radicals and antioxidant activity of flavonoids is highly dependent on the structure of the molecules that is the total number and position of hydroxyl groups (Farkas et al., 2004). There have been proven that hydroxyl groups of flavonoids in which located in the third and fifth carbon atom increase the antioxidant activity to almost 65%. This also happens to silymarin where hydroxyl groups also locate in the third and the fifth carbon atom of silybin, slychristin and silydianin. Besides that, all of those compounds have an extra hydroxyl group in the seventh carbon atom which could also contribute to the improvement of antioxidant activity (Kiruthiga et al.,2007).

The study of antioxidant characteristic in silibin has been evaluated to study the capacity to respond to ROS or other oxidants (Detaille et al., 2008). Some studies have

reported that milk thistle was not a great O_2^- scavenger and even more there was no reaction towards H_2O_2 (Abenavoli, 2010). However, Kiruthiga, in his research claims that oxidative stress from H_2O_2 in human erythrocytes was protected by silymarin extracts (Kiruthiga et al., 2007). Nonetheless, other research claimed that silymarin reacted with hydroxyl radical very quickly (Feher et al., 1988; Pradhan and Girish, 2006).

Activity silymarin against lipid peroxidation also has been reported many times. One report showed silymarin hinder linoleic acid peroxidation (Fiebrich et al., 2002) and another report published the capacity of silymarin to rat liver mitochondria induced by several lipid peroxide agents (Bindoli et al., 1977). Scavenging the free radicals is not only the method of silymarin as an antioxidant but it also gives impacts on glutathione and superoxide dismutase related enzymes (Valenzuela et al., 1989)

Earlier reports reported silymarin improved the redox activity and total glutathione content of several organs (intestine, liver, and stomach) in rats (Muriel and Mourelle, 1990). In addition, study of Kiruthiga in 2007 stated silymarin also showed enhancing the detoxification in which it acted preventing the depletion of glutathione (Kiruthiga, 2007). In 2009, Singh et al. published their report on how silymarin affects on reduced GSH and superoxide dismutase. They used acetaminophen as the free radical agent and exposed it to rats at toxic doses. The result indicated that silymarin reduced the free radical compounds by showing the improvement in reduced glutathione and superoxide dismutase. Other experiments of detoxification demonstrated by the activity of silibin to lessen the toxic effects towards damaged simian kidney cells induced by vincristin, ciplastin and paracetamol (Singh et al., 2009).

2.5 Ginkgo biloba

Ginkgo biloba, as also known as *Salisburia adiantifolia* or *Salisburia biloba*, is a valuable gymnosperm tree from Ginkgophyta division that has been used for mankind for more than 2000 years (Zhou and Zheng, 2003). Ginkgo biloba is deemed as 'living fossil' because it is the only one of plants from Ginkgophyta division that still survives. Although, the plants are widely spread in China, Japan and Korea, it is believed Ginkgo biloba is native to Zhejiang province in China (Beek et al., 2009).

Figure 6. Gingko biloba

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2.5.1 Ginkgo biloba as antioxidants

Ginkgo biloba has been planted and used as traditional medicines, mainly for asthma and bronchitis, in China for centuries ago (Kleijnen and Knipschild, 1992). In the 1730s Gingko biloba was firstly introduced to Europe (Singh et al., 2008) and the use of ginkgo leaf extracts for the benefit of blood circulation taken place in the 1960s in Germany. Presently, it is broadly used as a treatment for many diseases, namely cerebrovascular and peripheral problems, short-term memory, cognitive and neurosensory problems, ageing, dementias, tinnitus, and concentration problems. (DeFeudis et al., 2003). Even today gingko is also used as an optional treatment for Alzheimer's disease (Beek, 2009). In recent studies, the potency of extracted ginkgo biloba as being an antioxidant and radical scavenger in animal cells has been disclosed. (Liu et al, 2009).

Gingko leaf extracts that are usually used as medicines contain 3 active compounds, those include flavonoid glycosides (22-27%), terpene lactones (5-7%) and Ginkgolic acids (<5mg/g). (DeFeudis, 1998). Among them, flavonoid becomes the most important substance to give the antioxidant activity against free radicals (Goh et al., 2003). There are currently more than 30 authentic flavonoids found in ginkgo biloba. The high number of diverse flavonoids is due to the outcome of different glycosides in ginkgo (Beek, 2000). The flavonoids themselves consist of numerous number of polyphenol groups, including bioflavonoids, flavan-3-ols, flavonol glycosides, proanthocyanidins, flavones, acylated flavonol glycosides. Among other things, flavonol glycosides that are available in gingko leaves are more plentiful than the other flavonoids. Moreover, since the antioxidant activity of flavonoids will be highly contributed to the chemical structure and positioning of hydroxyl groups, some of flavonoids are better to be antioxidant agents than others. For instance, fisetin, quercetin, morin, and myricetin that are categorized as flavonoid aglycones known to have a better antioxidant properties than flavones (e.g. diosmetin, apigenin) and flavanones (hesperin and naringenin) due to the absence of 3-OH group (Singh, 2008). A prior research focused on comparing antioxidant activity among two extracts from different colour (yellow and green) of gingko. The results indicated that antioxidant activity in yellow leave extracts was stronger because of the richer content of flavonol aglycones (Kobus et al.,2014)

G. biloba trees secrete a diverse range of secondary metabolites and terpene trilactones (TTLs) are a secondary metabolite that is only present in G. biloba. Of all the compound groups, TTLs have received the biggest attention because they are the sole

secondary metabolites that own t-butyl group in the structure (Kobus, 2014; Beek, 2000). Two of most important TTLs that have been examined are ginkgolides (diterpenes) A, B, C, J and M, and a bilobalide (sesquiterpene) (Carrier, 1998; Singh, 2008). Ginkgolides A, B, C can be found in both roots and leaves, ginkgolides J can be only present in leaves whereas ginkgolides M exists only in the root. In 2001, Wang et al. revealed the structures of another ginkgolides, which are ginkgolides K and L (Beek, 2000). The structures of ginkgolides are cage-like containing carbocyclic rings (Singh, 2008) and only differ in their number and position of hydroxyl groups (Scholtyssek, 1997) the A, B, C, J and M can be seen in figure 7. While the structures of ginkgolides K, L and a bilobalide are shown in figure 8.

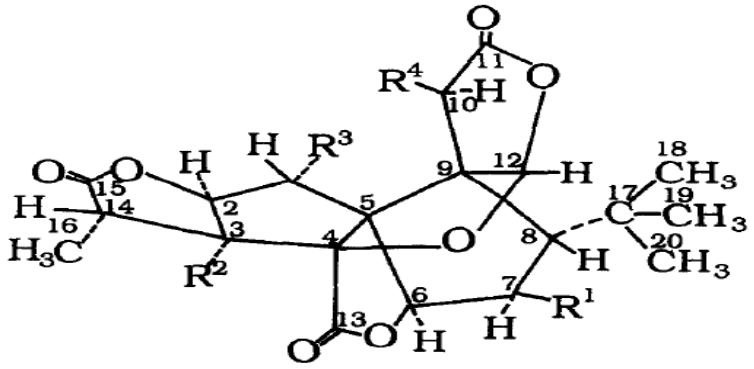
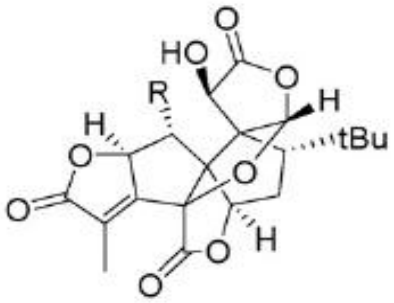


Figure 7. The structures of ginkgolides A, B, C, M, and J (Scholtyssek, 1997).
The explanation of R1-R4 can be seen in table 1.

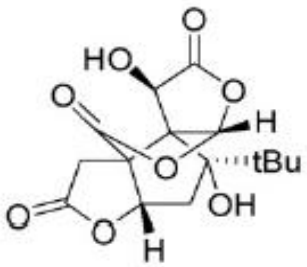
Table 1. Numbers and positions of hydroxyl groups of ginkgolides A, B, C, M, and J (Scholtyssek, 1997).

	R1	**R2**	**R3**	**R4**	**Compound**
1	H	OH	H	OH	Ginkgolide A
2	H	OH	OH	OH	Ginkgolide B
3	OH	OH	OH	OH	Ginkgolide C
4	OH	H	OH	OH	Ginkgolide M
5	OH	OH	H	OH	Ginkgolide J



G-K: R = OH

G-L: R = H



bilobalide

Figure 8. The structures of ginkgolides K (G-K), ginkgolides L (G-L) and bilobalide (Beek, 2009)

In 1997, Scholtyssek et al. reported their finding of ginkgolides as potent antioxidants. They stated that before their experiments antioxidative properties of ginkgo biloba extracts had mainly conducted in aqueous mediums (Scholtyssek, 1997). Ginkgolides, however, have exceptional solubility in hydrophobic environments, for instance, in lipids, where superoxide can stay longer because of the lack of protonation (Braquet, 1987). For this reason, the experiment was done in lipophilic environment. The results demonstrate that the ginkgolides B, C, M and J along with bilobalide were able to react with superoxide but not in the case of ginkgolide A. They claim ginkgolides C, B, M and J are potent superoxide scavenger in a lipophilic medium (Scholtyssek, 1997).

Ginkgo biloba might also help prevent lipid peroxidation by enhancing gluthatione and antioxidant enzymes. In the experiment of isoproterenol-induced myocardial necrosis in rats run by Panda and Naik exhibited the efficacy of 200 mg/kg treatment ginkgo biloba phytosomes in improving the depleted glutathione. They claimed, the improvement was either caused by the enhanced synthesis or the rise of gluthatione reductase level in the presence of ginkgo biloba phytosomes (Panda and Naik, 2008). In 2009, Panda and Naik conducted similar experiment and the result indicated the GSH reduced by ISO were brought back to normal by ginkgo biloba administration. In addition, the activity of antioxidant enzymes SOD, CAT and GPx were protected from depletion (Panda and Naik, 2009).

Huang et al. in their experiment inform ginkgo biloba extract can be extremely helpful in protecting body from free radical damages due to its special ability in interrupting oxo-ferryl radical species that is free radicals have greater oxidative effect than

mere hydroxyl radicals. Same effects of ginkgo biloba also demonstrated in their experiment (Huang et al., 2005).

2.6 The impact of milk thistle/silymarin and ginkgo biloba on blood profile.

Blood is a fluid that comprises of a vast array of substances, both organic and inorganic which has main functions for transporting oxygen, nutrients and enzymes to cells as well as discharging wastes (Casiday et al., 2000). Diseases or disorders in animals' health will affect on changing the blood properties. Thus, analysing haemotological and biochemical parameters is particularly important because many diseases or disorders will be revealed by examining the changes of the blood properties.

Without knowing the blood properties, sick animals may look healthy and conceal the clinical features. In other words, overall health status of animals can be detected by knowing the blood properties levels. Since one of the most important role of blood in the body is as a transporter, abnormality of organ functions such as in liver and kidney can be detected. There are some factors that influenced the consistency of those parameters which are cardiac rhythm breed, gender, age, feeding, disorders, environmental conditions, pregnancy and stress (Özkan et al., 2012). This table below show general reference to the range of normal level of blood biochemistry in rabbit blood.

Table 2. Ranges of normal level of some blood biochemistry in rabbit blood (Suckow et al., 2011)

Biochemistry	Range	Biochemistry	Range
Glucose	75-155 mg/dl	Total Protein	5.4-8.3 g/dl
Total Cholesterol	10-80 mg/dl	Globulin	2.4-4.6 g/dl
ALP	10-140 IU/l	Albumin	1.5-2.8 g/dl
ALT	14-80 IU/l	Calcium	5.6-17 mg/dl
AST	14-113 IU/l	Phosphorus	2.3-6.7 mg/dl
Amylase	200-500 IU/l	Total bilirubin	0-0.8 mg/dl

2.6.1 The impact on liver transaminases (alanine aminotransferase and aspartat amino transferase) and alkaline phosphatase.

Alanine aminotransferase (ALT) and Aspartat aminotransferase are marker enzymes to determine the physiological state of the liver. It is due to the great response of the enzymes to unbalance state of the liver, once the liver has been damaged, ALT, AST and ALP release into circulation so the concentrations of those enzymes will then increase (Shaarawy et al., 2009). This makes ALT and AST as very reliable indicators of liver physiological state. ALT has a function in catalyzing the formation of pyruvat and glutamate. While AST function in catalyzing oxaloacetate and glutamate from aspartate and α-ketoglutarate.

Similar function as liver transaminases, alkaline phosphatase also changes when in liver is an abnormal state. It will monitor the not only the physiological state of liver but also bones. The result of Dr. Anurag Jain's trial in hepatotoxicity albino rabbits in 2013 showed ALT, AST and ALP decreased after the prior increase post inducement of CCL4 hepatotoxic agent (Jain et al., 2013).

In Muhammad Habib-ur-Rehman et al. study of the effect of silymarin on ethanol induced hepatotoxicity also demonstrated silymarin could normalize liver function by reducing ALT from ±82 (U/l) to ±49 (U/l) after skyrocketed increase from ±28 (U/l) in the normal state. This occurrence showing that silymarin has been successfully minimized the leakage of enzymes by maintaining integrity of cells. This leakage of enzymes apparently caused by protein oxidation, lipid peroxidation and reactive oxygen species (ROS) which was due to the ethanol inclusion resulting in hepatocytes damage (Habib-ur-Rehman et al., 2009). Similar results of silymarin as hepatoprotective agent recovering the abnormal rise of ALT, AST and ALP also have been reported by many researches (Ilyas et al., 2011; Abu-zaiton, 2013; Rasool et al, 2014; Oda and El-Ashmawy, 2012; Shaarawy, 2009; Ramadan, 2002; Ghaffari et al., 2011).

Besides the effect of hepatoprotection Oda and El-Ashmawy also conducted a series of trial of silymarin effect on healthy rats. The level of ALT, AST and ALP did not differ significantly from those of controls (Oda and El-Ashmawy, 2012). It was confirmed by other exact the same result of Sakr experiment which was shown no significant difference of both ALT and AST in comparison to control post silymarin inclusion (Sakr and Al-amoudi, 2012)

Shenoy et al, examine the hepatoprotective effect on both silymarin and ginkgo biloba individually. They claimed two of them are very potential to give protection against hepatotoxicity since the results of them were identical. There was a marked decrease in ALT, AST and ALP level after inclusion of silymarin and ginkgo biloba respectively (Shenoy et al, 2001).

In contrast, in the experiment of Tedesco et al. on the efficacy of silymarin in reducing the toxicity of aflatoxin showed ALT declined considerably after broilers administered with aflatoxin and Silymarin bring ALT value back to its normal state. The decline in this experiment can be understood since the impact of hepatotoxic agents are not always the same, in this case it was due to aflatoxin intoxication in broilers (Tedesco et al., 2004).

Likewise silymarin, there are many researchers concluded their results that ginkgo biloba could minimize the negative effect of liver toxicity by reducing the level of ALT, AST (Zhou et al., 2010; Huang et al., 2005; Şener et al., 2006; Şener et al., 2007; Yao et al.,2007; Þener et al., 2005; Şener et al., 2006; Chang et al., 2007, ALP (Parimoo et al., 2014; Naik and Panda, 2008; Ye et al., 2011)

2.6.2 The impact on total bilirubin (TB)

Many evidences have proved silymarin to decrease total bilirubin level after increase due to harmful condition. The study of hepatoprotection of silymarin titled "Protective Effects of Silymarin in Isoniazid Induced Hepatotoxicity in Rabbits" revealed the addition of silymarin might increase the bilirubin level, but it could also protect the rabbits from the excess of bilirubin so called hyperbilirubinaemia. When silymarin was introduced to healthy rabbits for 12 days, there was 0.08 mg/dl increase from 0.28 mg/dl to 0.36 mg/dl.

Hepatotoxicity drug boosted the level of bilirubin from 0.33 mg/dl to 0.5 mg/dl and administration of silymarin normalized it to 0.31 mg/dl (Maryam, 2010). Normalization of bilirubin level also can be seen in study of Ghaffari et al. on hepatic fibrosis rats (Ghaffari et al., 2011).

Comparable result was shown in applying ginkgo biloba on hepatic fibrosis rats which was also prevent the destruction of bilirubin and rebound its level back to normal. However, there was no significant difference between control group and ginkgo biloba inclusion group of normal rats (Þener et al., 2005).

2.6.3 The impact on total protein (TP), globulins (GLOB) and albumin (ALB)

Supplementation of milk thistle seed on broiler feed was investigated by Nik et al. In his research, supplementation of milk thistle seed alone could increase total protein, globulin and albumin levels, however, significant results were performed by using combined milk thistle and thyme seeds (Nik et al., 2014).

The reduction of albumin due to the inclusion of hepatotoxic agent was considerably enhanced by silymarin and even normalized. (Jain et al.,2013; Ghaffari et al., 2011). Extract milk thistle also increased albumin level of diabetic rats (Abu-zaiton, 2013). A peculiar outcome has been exhibited by Mansour et al. that albumin level tended to lower considerably after silymarin addition. In spite of this reduction, silymarin worked well in securing level of albumin in intoxicated rats which was higher than that of only ciplatin-induced rats (Mansour et al., 2006).

In 30-day trial of silymarin extract on rainbow trout showed significant increases on total protein, globulin and albumin (Ahmadi et al., 2012). However, Oda and El-Ashmawy stated there was no difference of concentrations between total protein and globulin of healthy control rats and mercury-induced intoxicated rats while only a slight increase occurred in the level of albumin (Oda and El-Ashmawy, 2012).

In the study of El-Shafeety milk thistle could increase the albumin and protect it from declining due to toxicity, but the level of albumin would when it was combined with grape seed in intoxicated fumonisin rats (El-Shafeety et al., 2012). Another significant increase of total protein was shown in the research on hepatoprotective of silymarin towards acethylsalysilic acid but there was no effect on alanine aminotransferase and bilirubin (J Kucharz and Kott, 1994). Similarly, the increase of protein after being exposed to the hepatotoxic agent shown by Rasool et al (Rasool et al., 2014).

In the research of hepatoprotective Ginkgo biloba conducted by Parimoo et al., inclusion of hepatotoxic lantadenes in guinea pigs resulted in substantial increase in total protein level and ginkgo biloba decreased it (Parimoo et al., 2014). On the other hand, rifampicin-induced hepatoxicity in rats indicated a dramatic decrease in total protein level, the use of ginkgo biloba (50 mg/kg p.o) or silymarin (100 mg/kg p.o) could restore it back to normal (Naik and Panda, 2008). Similar experiment and result was demonstrated in CCl-induced rats by Yang, not only protein, however, decreased albumin level was also improved by addition of ginkgo biloba (Ye et al., 2011).

Shui-Xiang He et al. carried out 8-week trial to find out the effect of ginkgo biloba on liver injured rats. They found that 0.5 g/kg body weight per day of ginkgo biloba had increased significantly the level of bilirubin after previously being lowered by Carbon tetrachloride (He et al., 2006).

Shenoy et al also proved both silymarin and ginkgo biloba could reverse the declined of total protein and albumin level caused by hepatotoxicity agent (Shenoy, 2001).

2.6.4 The impact on calcium (Ca) and phosphorus (PHOS)

Calcium and phosphorus play great role in the formation of teeh and bone as well as regulation of kreb cycle. In 2009 Nagla A. El-Shitany et al. carried out experiment on extrogenic effects in silymarin. The experiment involved 10 rats and 50 mg/kg/ day of silymarin. They concluded that the level of calcium and phosphorous in serum was influenced by silymarin addition. They observed significant increases in calcium and phosphorous,±2 mmol/l and ±1.5 mmol/l respectively, on ovariectomized rats over that of without any silymarin addition (El-Shitany et al., 2010). In contrast, a result of Mansour et al. indicated 100 mg/g/day silymarin reduced remarkably the concentration of calcium from 15.81 to 7.85 mml/l (Mansour et al., 2006).

2.6.5 The impact on glucose (GLU)

In 2013, Ahmed Saber conducted experiment to evaluate the effects of *Silybum marianum* extract on blood glucose level, cholesterol, liver enzymes and kidney functions in diabetic rats. The result was a marked reduction in glucose level about 33% at dose 200 mg/kg (Abu-zaiton, 2013).

In the study of the hypoglycaemic activity of *Fraxinus excelsior* and *Silybum marianum* in 2004, Maghrani et al. administered orraly 20 mg/kg aqueous silymarin extract to both normal and diabetic rats. Glucose level was decreases in both normal and diabetic rats, however, the effect rate and efficacy of silymarin were greater in diabetic rats (Maghrani et al., 2004). A significant reduction of glucose also occurred in hyperglycemic rats treated with silymarin yet the efficacy was higher in the mix of silymarin and burdock (*Arctium lappa*) indicated from returning back glucose concentration to normal (Bakr and ElSawy, 2014)

2.6.6 The impact on amylase (AMYL)

Amylase is an enzyme produced in pancreas which breaks down complex carbohydrates to small form monossacharides. A reduction of 12% amylase after addition of 200 mg/kg milk thistle extract in diabetic rats was reported (Abu-zaiton, 2013). In the study of Zeybek et al. on experimental acute pancreatitis, serum amylase of pancreatitis rats was decreased substantially after pretreatment with ginkgo biloba even though it did not return back to normal (Zeybek et al., 2003).

2.6.7 The impact on cholesterol (CHOL)

Silymarin also gave full protection against adverse effect of increase in the ratio of cholesterol : phospholipids (Jain et al., 2013). There was a restoration of cholesterol level of diabetic rats after *Silybum marianum* being fed at dose 400 mg/kg (Abu-zaiton, 2013).

Ansari nik et al. reported that *Silybum marianum* seeds significantly lessened cholesterol, triglycerides, and LDL (Low Density Lipoprotein) cholesterol concentration in serum of broiler chicks. Moreover, the combination of *Silybum marianum* and *Thymus vulgaris* could give better result and even improved HDL (High Density Lipoprotein) cholesterol considerably (Nik., 2014).

Another strong evidence that silymarin reduced cholesterol level is shown in El-Shafeey et al. study. The addition of milk thistle extract could return the level of total cholesterol which was increased by inclusion of fumonisin. It was also discovered that LDL, HDL, and TG (triglycerides) had been reduced over the usage of milk thistle. They also found that the combination between Milk Thistle and grape seed extract had given synergistic effect of lowering cholesterol level (El-Shafeety et al., 2012).

Positive effect of ginkgo biloba in reducing cholesterol and triglyceride levels has been reported by Yang et al. They concluded that ginkgo biloba has no different efficacy in reducing cholesterol and triglyceride that of commercial hepatoprotective drug, Essentiale, as both of them could restore the level to normal (Ye et al., 2011).

In the experiments with hyperlipideamic rats, cholesterol levels in plasma and LDL cholesterol can be reduced by the administration of silymarin. However, in normal rats, silibyn can only reduce phospholipid levels, but not decrease plasma levels of cholesterol (Skottova and Krecman, 1998)

2.6.8. Urea (UREA)

Urea is an important parameter for detecting kidney function. Silymarin has demonstrated its capability in protecting kidney against harmful damage from many sources including platinol, oncovin and acetaminophen it is thought mainly from lessening lipid peroxidation (El-Shafeety et al., 2012). In the investigation of efficacy extract silymarin at dose 200 mg/kg and 400 mg/kg, it was found that the former dose had a greater effect on reducing the urea concentration in diabetic rats (Abu-zaiton, 2013)

In Oda and El-Ashmawy experiment, silymarin at dose 200 mg/kg made substantial reduction in urea level of nephro-hepatotoxicity in rats. Nevertheless, there was no difference between control and silymarin-fed healthy rats (Oda and El-Ashmawy, 2012). Higher dose at 600 mg/kg silymarin was given in hepatic fibrosis rats due to methotrexate and silymarin could return the urea level back to normal (Ghaffari et al., 2011).

In contrast, there was no significant result of lowering the urea level in fumonisin induced rats in El-Shafeety study (El-Shafeety et al., 2012).

A huge increase occurred in the urea level of rats after 72-hour irradiation exposure. Ginkgo biloba deliver potential benefit to reducing the urea level showing the return of the level back to normal (Şener et al., 2006).

2.7 The impact of silymarin and ginkgo biloba on feed intake and body weight

In 2013, Dr. Anurag Jain conducted a research on the hepatoprotective effect of Silymarin by inducing CCl4 on rats. The experiment demonstrated silymarin could improve significantly feed intake as well as body weight under CCl4 inducement (Jain et al., 2013).

In the study of Silymarin-Phospholipid Complex in Reducing the Toxicity of Aflatoxin B1 in Broiler Chicks, Tedesco et al. claimed silimaryn could have a constant and positive effect in counteracting the drawback of Aflatoxin on feed intake and body wright. This was due to the return of the feed intake value of the Aflatoxin addition to its control (Tedesco et al., 2004). The usage of Milk thistle could exhibit more superior efficacy in improving feed intake and body weight after an unfavourable condition when it was utilized along with burdock (*Arctium lappa*). A Dosage 500 mg/kg milk thistle extract could improve feed intake significantly while 500 mg/kg mix of milk thistle and burdock could return the value back to normal. The same result also occurred in the body weight gain (Bakr and ElSawy, 2014).

The importance of ginkgo biloba to body weight and feed intake has also been studied many times. Ginkgo biloba could raise body weight and food intake and ameliorate the impact of ethanol after 90-day treatment in rats (Yao et al., 2007).

The experiment of feeding whole ginkgo biloba nuts showed promise in increasing feed intake (Mahadevan et al., 2008).

Another experiment, Ginkgo biloba could also increase the weight of aging rats and correct the proportion of the ratio between liver and body weight (Huang et al., 2005).

2.8 Overview of rabbits

2.8.1 Digestive system

The digestive systems of rabbits allow them to have a high feed intake (65–80 g kg−1 body weight) and process digestible and easily fermentable nutrients quickly by separating them from low digestible waste which are then disposed rapidly. This separation was because they have substantial caecum and colon, which make them differ from other species. This special activity possible them to not require a wide absorption surface area in the large intestine. The proportion of cecum in rabbit is the biggest among mammals. It is two folds longer than abdominal cavity and accounts for of up to 60% of the gastrointestinal tract. The high content of cecal microorganisms plays great roles in the digestion (Davies and Davies, 2003; Blas and Wiseman, 2010).

When the cecum of rabbits is compared to rumen in mammals, the content of protease and amylase are higher but cellulase is lower (14% in rabbits while 44% in cattle). This leads to the lesser amount of the utilization of fibre but it will increase as rabbits grow. In addition, rabbits also have poor inulase and β-glucosidase in their gastrointestinal tract (Yu et al., 2000; Irlbeck, 2001). Before the digestive system of rabbits is fully functional, they must be fed by milk-based diet to give the adaptation to the cecal microorganism (Blas and Wiseman, 2010).

2.8.2 Feeding and housing

Apart from having a high feed intake, rabbits also possess fast feed transit rate to meet their nutrient needs. Some amino acids are limiting in the traditional feeding practice, usually lysine and methionine. The coprophagy (ingesting its soft faeces) behaviour of rabbits can also enhance protein digestibility. Volatile fatty acids may serve some energy for maintenance, however, different from those of ruminants, starch will generate more volatile fatty acids in which acetate is the primary production. Fiber is very important for

rabbits particularly to preserve gut health and encourage gut motility. Oats and other low-energy based feed are more preferable than high-energy feed like starch due to incomplete absorption and leading to enteritis. Enteritis may also occur when rabbits are fed by finely ground feed. Rabbits have a capability to absorb calcium without being facilitate by vitamin D. Alfalfa based diets usually contain high calcium, excess calcium may trigger abnormality in liver. In order to prevent gastrointestinal discomfort, a decent fiber concentration (20-25%), low startch and proper amount of protein feed should be given (Irlbeck, 2001). In a great management breeding practice, rabbits may convert protein into meat better than pigs or cattle, up to 20% while only up to 18% in pigs and 12% in cattle (Nistor et al., 2013).

Environmental factors can affect on nutrient intake of rabbits. Rabbits which are kept in the dark environment may have higher feed intake than those giving normal daily light. Stocking density will have a great influence on feed intake. Having too many rabbits in a cage will cause rabbits cannot eat freely due to high competition. Temperature needs also be taken into consideration. Rabbits cannot stand very cold or very hot condition, they may suffer from heat stress if the temperature is more than 30°C (McNitt et al., 2013).

3 Aim of the thesis

- Main objective is to compare the biochemical properties in rabbit blood when fed with or without AV3.
- Partial objective :
  - To compare the growth performance when fed with or without AV3
  - To compare blood and liver antioxidant status (GSH) of rabbits when fed with or without AV3.

4 Hypothesis :

- There are differences in biochemical properties between AV3 and the control group.
- There is an improvement of the antioxidant (GSH) in rabbit blood in AV3 group in comparison to the control.
- Rabbits which are fed with AV3 will have better growth performances and lower mortality.

5 Material and methods

For the whole experiments, 180 rabbits HYLA broiler rabbits in total were used with two groups of feeding groups. 30 rabbits in the control groups and 30 rabbits in AV3 group were used per experiment. The rabbits were obtained from the farm of Mr. Kočár (Genetic Center HYLA in Ratibořice, CR). The rabbits were brought into CULS Prague experimental stable at the age of 35 days. The experiment was begun after 7-day acclimation. Male and females were randomly divided into 2 groups (AV3 group and control group) and moved into enriched cages (n=3 rabbits/cage). There was a free access to water in each cage and *ad libitum* feeding was given. Standard feed mixture with anti coccidia (EMANOX PMX) and probiotic (PROBIOSTAN E10) were given as a control group. AV3 with dose 0.20 ml/kg was added in control mixture as the feed for AV3 group. The feed mixture was produced by Biokron Ltd. Company while the AV3 was supplied by Manghebati SAS, France. The exact concentrations of each substrate in AV3 were not disclosed by the company. However, the active substance of silibyn content was 25.2 mg/l. The dosage of AV3 feed was in respect to the recommendation of the manufacture.

The experiment was done in the temperature at 18-20°C with three repetitions. Body weight (BW) was recorded weekly to calculate the average daily gain (ADG). Daily feed intake, feed conversion and total feed consumption were also recorded.

The experiment was ended after the rabbits had reached 2600g with 84 days of age as a limit. There were 10 samples of blood and liver tissue from each group in each experiment collected. Slaughtering process began with stunning animals by captive bolt in the head. Slaughtering process was done by cutting on the carotid artery causing bleeding, then the shed blood was collected. Serum was gathered by filling the blood into 3 mm VACUETTE® test tubes with blood activator added. Liver, kidney and carcass were weighed as well. The total increase of weight was done by subtracting the initial weight from slaughter weight. Liver and serum were brought into a laboratory of faculty Tropical Agrisciences for further analysis. Serum was used for examining biochemical properties and the liver was kept in the fridge (-20°C) in order to be sent in University of Veterinary and Pharmaceutical Sciences Brno (VFU Brno) for liver analysis.

The whole research was accomplished in one year in 2014. Serum was immediately assayed for the contents of biochemical properties in the laboratory of Department of Animal Science and Food Processing, CULS Prague. From 3 mm VACUETTE® test tubes, serum was moved into 0,5 millilitres Eppendorf® tubes. Serum

was then centrifuged with 10,000rpm in 3 minutes. Supernatant was collected and assayed with advanced blood tested machine, VetTest Analyzer (IDEXX Laboratories). The biochemical properties assayed were albumin (ALB), alanine aminotransferase (ALT), alkaline phophatase (ALP), glucose (GLU), amylase (AMYL), cholesterol (CHOL), globulin (GLOB), calcium (Ca), phosphorus (PHOS), total protein (TP), asparate aminotransferase (AST), and urea.

Antioxidant detection was done by examining the reduced glutathione (GSH) level in the liver. Liver tissue firstly was homogenized and the supernatant was collected to determine GSH by using Ellman method based on spectrophotometric as GSH can create complex color upon reaction with Ellman's reagent (5,5'-dithiobis-2-benzoic acids or DTNB) at a wavelength of 412 nm.

Statistical evaluation of the biochemical properties and growth performance parameters was done using GLM (General Linear Model) Procedure in SAS System V 9.3. While the statistics of the GSH level was done in excel by using two-tailed student t-test.

6 Results

This research was in cooperation with University of Veterinary and Pharmaceutical Sciences Brno (VFU Brno). Some results of biochemical properties, reproductions and reduced glutathione from the dams of our experimental rabbits examined by VFU Brno were included as comparisons.

6.1 Biochemical properties

6.1.1 Serum levels of alanin aminotransferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP) and amylase (AMYL).

Based on statistics on GLM results, there is no significant change of liver enzymes (ALT, AST and ALP) and pancreatic enzyme (AMYL) in AV3 group in comparison to the control group (P>0.05).

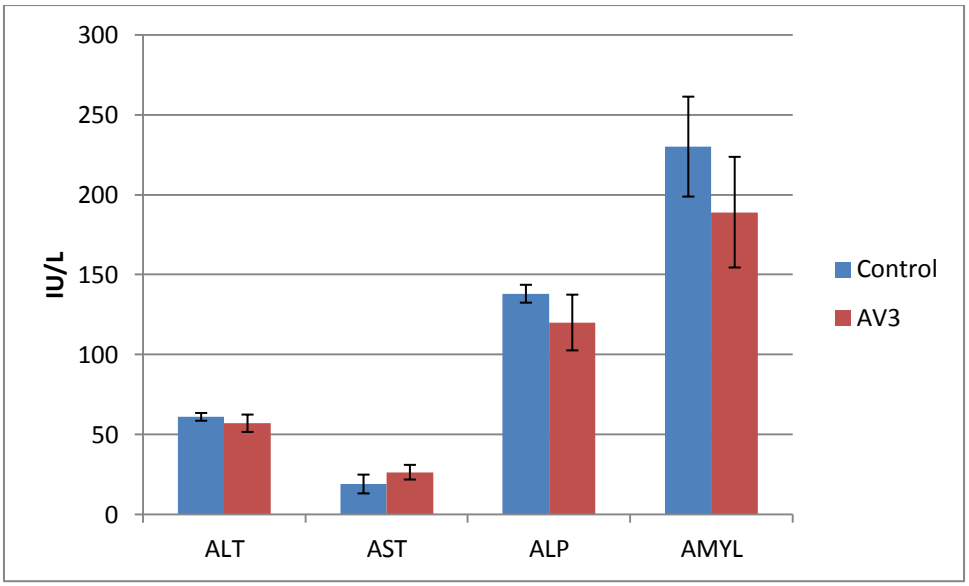


Figure 9. Serum activity levels of liver enzymes (ALT, AST and ALP) and pancreatic enzyme (AMYL) (mean+SD).

6.1.2 Serum levels of total protein (TP), albumin (ALB) and globulin (GLOB)

Based on statistics on GLM, there is no significant change of total protein, albumin and globulin levels in AV3 group in comparison to the control group (P>0.05).

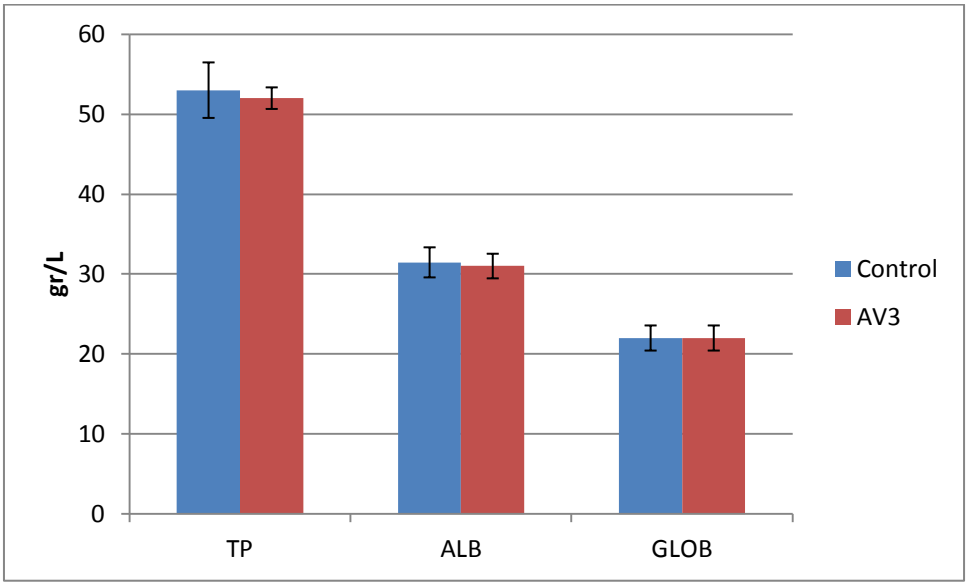


Figure 10. Serum activity levels of total protein (TP), albumin (ALB) and globulin (GLOB) (mean+SD).

6.1.3 Serum levels of calcium (Ca), cholesterol (CHOL), phosphorus (PHOS), urea and glucose (GLU)

Based on statistics on GLM, there is no significant change of calcium (Ca), phosphorus (PHOS), urea and glucose levels in AV3 group in comparison to the control group (P>0.05). Whereas there is a significant increase in cholesterol level (P=0.0086) by 38.6% in the AV3 group when compared to that of the control group.

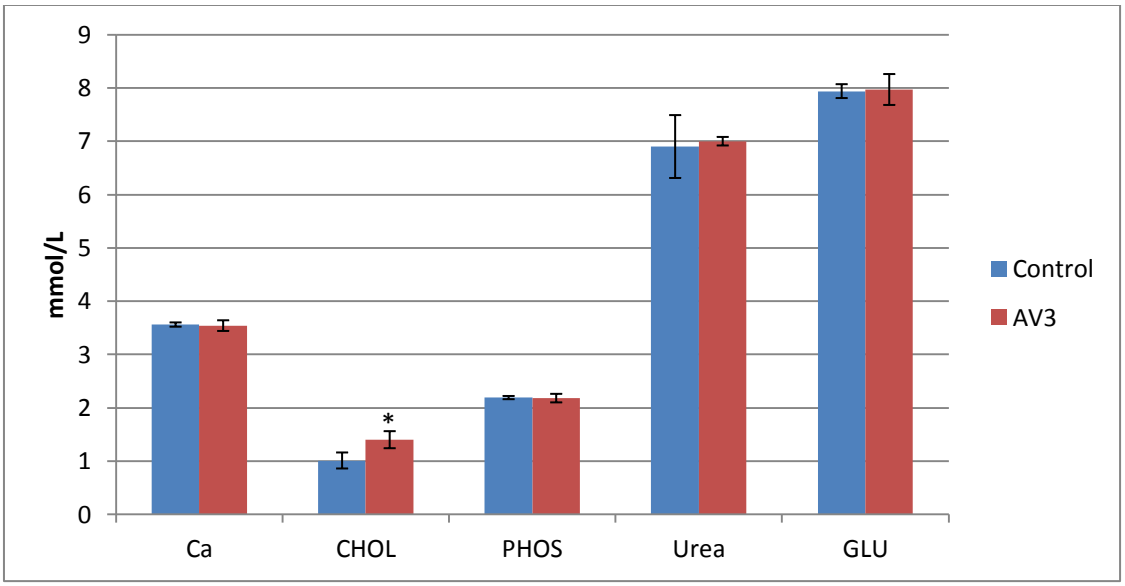


Figure 11. Serum activity levels of calcium (Ca), cholesterol (CHOL), phosphorus (PHOS), urea and glucose (GLU) (mean+SD). *P<0.05.

Table 3. Table comparison of biochemical properties (min, max and mean) between control and AV3 group.

Biochemical	**Control**		**AV3**	
	Min - Max	**Mean + St. Dev**	**Min**	**Mean + St. Dev**
ALT (U/l)	34 – 91	61±2.44	38 – 76	57±5.43
AST (U/l)	9.33 – 36.67	19.46±5.88	4.33 –47.67	26.39±4.58
ALP (U/l)	93 – 232	138±5.59	80 – 187	120±17.43
TP (gr/l)	46 – 58	53±3.47	39 – 59	52±1.35
ALB (gr/l)	26 – 35	31.46±1.88	28 – 34	31±1.54
GLOB (gr/l)	19 – 25	22±1.57	19 – 27	22±1.57
AMYL (U/l)	136 – 372	230±31.25	118 – 248	189±34.6
CHOL(mmol/l)	0.45 – 1.88	1.01±0.15	0.92 – 1.96	1.4±0.16*
Urea (mmol/l)	6 – 8.6	6.9±0.59	5.3 – 9.3	7±0.08
PHOS(mmol/l)	1.79 – 2.45	2.19±0.03	1.93 – 2.42	2.18±0.08
Ca(mmol/l)	3.36 – 3.72	3.56±0.04	3.43 – 3.64	3.54±0.1
GLU (mmol/l)	7.35 – 8.69	7.94±0.13	7.38 – 8.7	7.97±0.29

*P<0.05

The comparisons of concentrations of albumin, ALT, AST, cholesterol and urea between our experiment (litter) and VFU Brno (Dam) are depicted between our experiment (litter) and VFU Brno (Dam) figure 12-16.

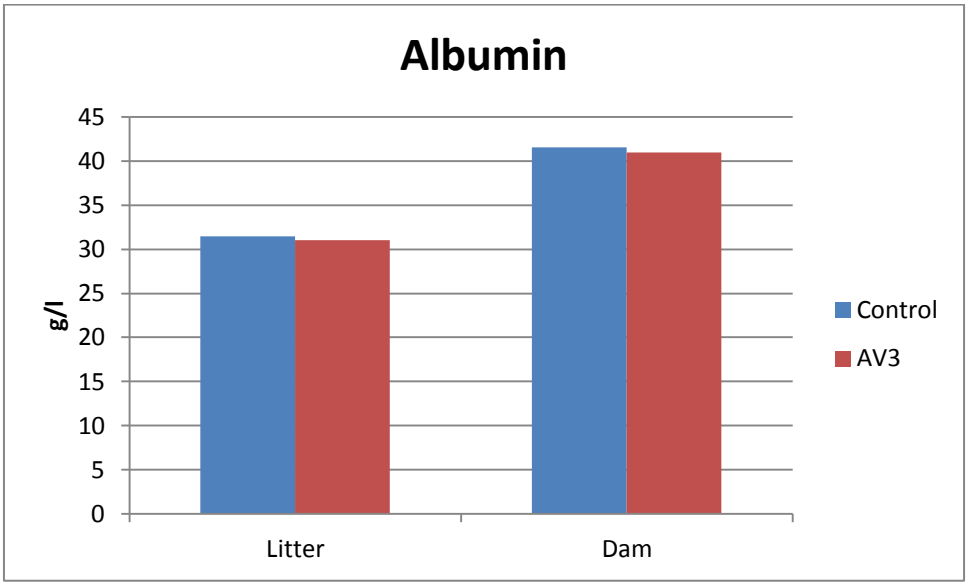


Figure 12. Difference concentration of albumin with or without AV3 between litter and dam group.

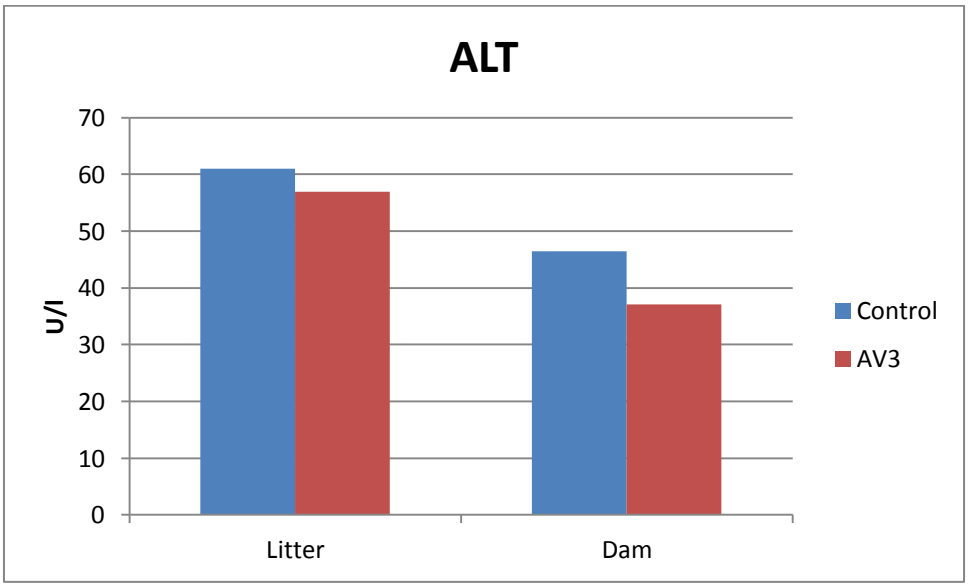


Figure 13. Difference concentration of ALT with or without AV3 between litter and dam group.

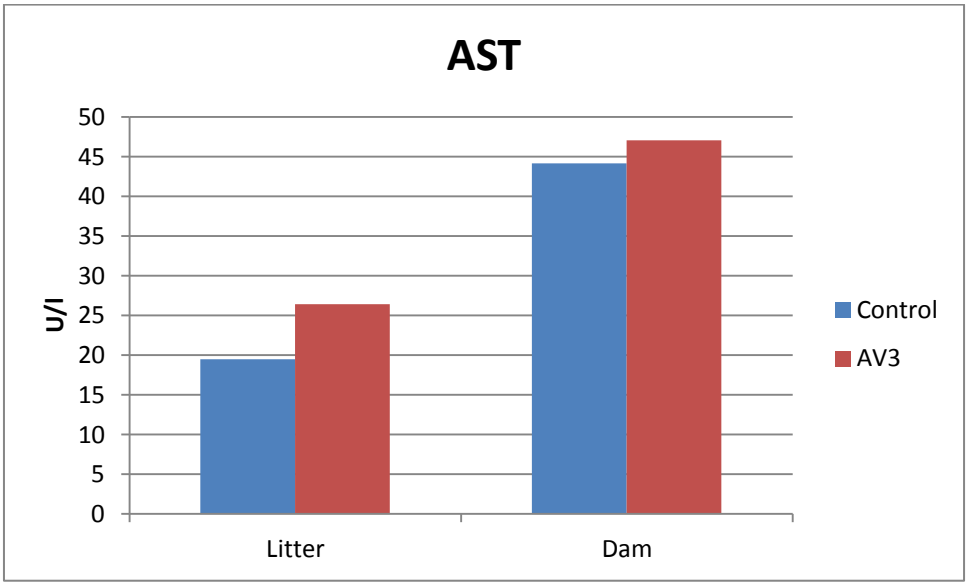


Figure 14. Difference concentration of AST with or without AV3 between litter and dam group.

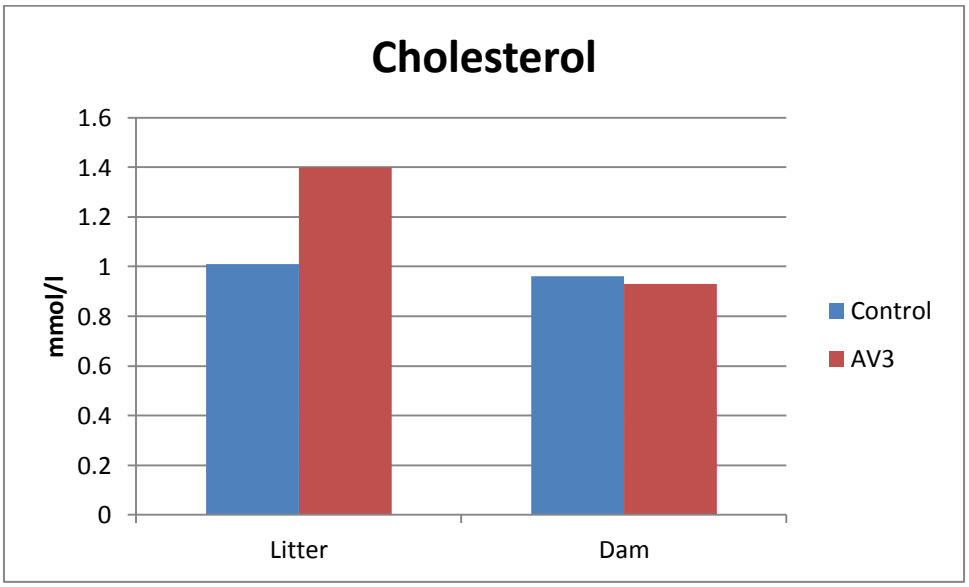


Figure 15. Difference concentration of cholesterol with or without AV3 between litter and dam group.

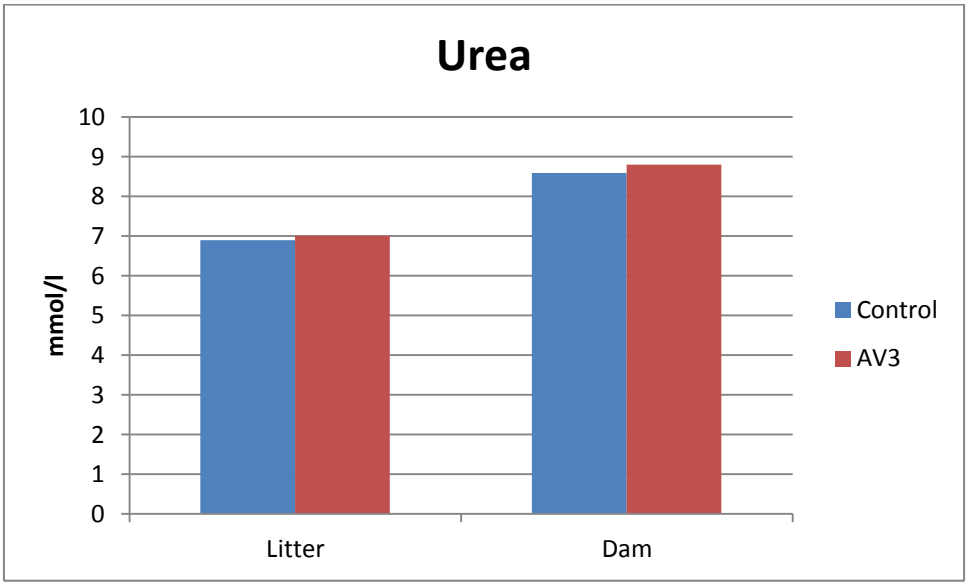


Figure 16. Difference concentration of urea with or without AV3 between litter and dam group.

6.2 Antioxidant reduced glutathione (GSH)

According to the result of t-test, GSH level AV3 group is not significantly different from that of the control group (P>0.05).

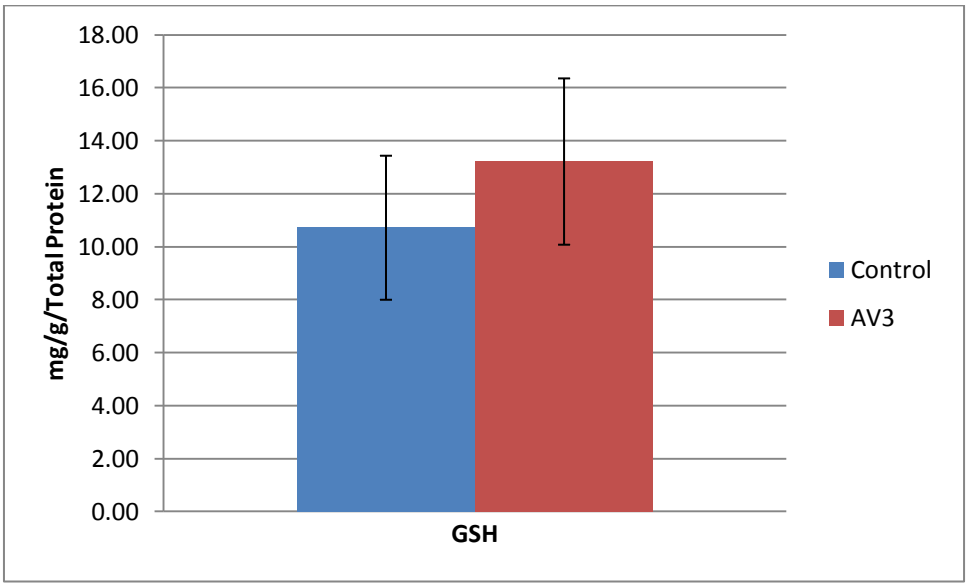


Figure 17. Comparison of GSH level between control and AV3 group (mg/g/total protein).

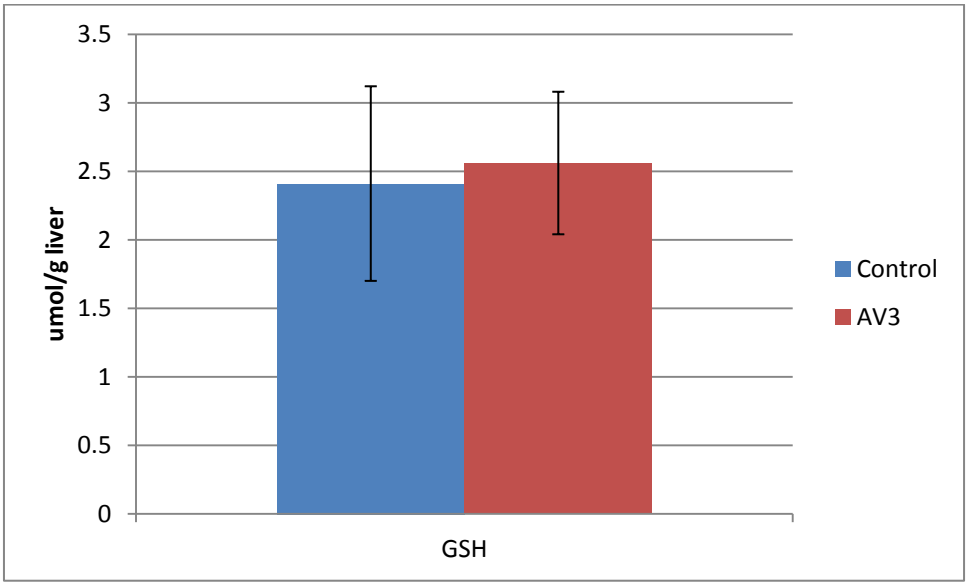


Figure 18. Comparison of GSH level between control and AV3 group (umol/g liver).

Table 4. Table comparison of biochemical properties (min, max and mean) between control and AV3 group.

Groups	**GSH (mg/g/total protein)**		**GSH (umol/g liver)**	
	Min – Max	**Mean + St.Dev**	**Min – Max**	**Mean + St.Dev**
Control	8.3 – 15.3	11.28±2.37	1.77 – 3.94	2.41±0.71
AV3	9.8 – 21.1	13.22±3.14	1.97 – 3.79	2.56±0.52

6.3 Growth performance parameters and mortality

There are no significant differences in most parameters ($P>0.05$) according to GLM test between the control and AV3 group. The addition of AV3 led to an increase in kidney weight by 8.1%. Whereas, carcass yield was also elevated by AV3 supplementation from 57.22% to 58.31%. Mortality was increased from 12% in the control group to 13.33% in AV3 group. The percentage of rabbits which exclude in the experiment because they cannot reach sufficient weight in 84 days was significantly higher ($P<0.05$) in AV3 group (22.22%) than that of control group (9.33%)..

Table 5. Table comparison of growth performance parameters between AV3 and control group.

Growth parameters	**Group**			
	Control		**AV3**	
	Mean	SE	Mean	SE
Slaughter weight (g)	2717.89	8.96	2722.57	13.68
Daily feed intake (g)	154.64	0.89	153.46	1.36
Daily gain (g)	40.84	0.35	40.64	0.54
Feed conversion (kg.kg-1)	3.87	0.04	3.85	0.06
Total weight increase (g)	1390.58	12.36	1404.84	18.87
Total feed consumption (g)	5368,11	33.2	5376.88	50.69
Liver weight (g)	94.54	1.7	98.62	2.6
Kidney weight (g)	43.1	0.92	46.59 *	1.4
Carcass weight (g)	1562.11	8.12	1590.55	12.4
Carcass yield (%)	57.22	0.28	58.31*	0.43

*P<0.05

7 Discussion

Rabbits were used in this experiment because they are easy to handle (timid) and they have short life cycles. We studied the benefit of AV3 in fattening rabbits for meat production. In this experiment indicated that the addition of AV3 as feed additive did not significantly affect on the biochemical properties except for cholesterol.

To the best of our knowledge, there is no prior research involves the addition of AV3 on rabbit feed besides the research at VFU Brno. They supplemented AV3 on the feedstuff of the dam of our experimental rabbit. Our results are in conjunction with their results indicating that most of the results were not significant. Similarly, there are also very limited resources on the use AV3 on other mammals. For this reason, the comparison between our results and prior researches was rather complicated, it was done mainly with MTE or GBE alone either on rabbits or other mammals.

Liver transaminases namely alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and alkaline phosphatase (ALP) are very important biomarkers in the detection of liver damage as they are discharged from cytoplasm into circulation after being damaged (Maryam et al., 2010). As can be seen in figure 9, the results of ALT and ALP were lower in a group fed by AV3 but there is no difference statistically. This decrease is similar to Shaarawy et al. study which the level of ALT and AST were slightly reduced in comparison to the control due to the addition of silymarin and garlic. These levels of ALT and AST were even lower than the groups that were given with silymarin or garlic alone (Shaarawy et al., 2009). In spite of the liver enzymes, the pancreatic enzyme, amylase, was also lower in AV3 group but statistically the same. The decreased amylase was below the minimum recommended value in table 2. However, the level is still higher than other experiment in healthy rabbit conducted by Burski et al., where the mean of amylase in healthy rabbits was 124 U/l. This is comprehenable since many factors influence the normal biochemistry value (Burski et al., 2004).

The reduction of ALT in AV3 group in litters and dams was also demonstrated in VFU Brno analysis with lower concentration in the dam group compared to the litter group. Whereas AST in the litters and dams increased in the AV3 group with higher concentration of AST in the dam group compared to the litter group.

It can be suggested that the slight changes of these liver and pancreatic enzymes might be influenced by the synergistic effects of those two plant extracts (MTE and GBE). MTE and GBE may try to maintain homeostasis by retaining some enzymes inside the

organs while releasing some other enzymes in the blood. However, since these insignificant levels, these changes may be overlooked. The increase of liver enzymes in the dams is particularly due to the aging process. Thise reason is in agreement with Dontas et al., who studied a time- or stress-effect changes in blood biochemistry and Matsuzawa et al., who have examined on age-related biochemical alteration in a multitude number of other animals (Dontas et al., 2011; Matsuzawa et al., 1993).

The detection of urea and creatinine was important to detect kidney failures. Our result indicates that there was no difference in urea level between those groups. Urea level in the dam examined by VFU Brno was shown having higher concentration than our experiment. Nevertheless, there is also no significant change to be detected in the dam group. In Şener et al. study, the involvement of GBE in undamaged tissue rats did not have a significant impact on blood urea nitrogen (BUN), creatinine as well as the liver biomarkers AST and ALT. Nevertheless, GBE could restore the abnormality of those biochemical factors after mercury chloride inducement (Şener et al., 2007). In the study done at VFU Brno, creatinine was enhanced significantly by AV3 inclusion. The result of the urea level of our study implies that there is no failure in the kidney function so AV3 would not disturb this normality of urea level.

Cholesterol level was raised significantly in AV3-fed group compared to the control. This rise does not necessarily mean the status of the animals was worse than the control. Cholesterol is needed in the body system of animals to synthesis vitamin D, hormones and many substances that assist in food digestion. As we know, there are two kinds of cholesterol, LDL and HDL, where synthesized in the liver. We did not conduct research on LDL and HDL cholesterol, instead we only analysed the total cholesterol level. Total cholesterol level includes LDL cholesterol, HDL cholesterol and triglycerides. High content of HDL cholesterol is desirable, in contrast, high content of both LDL and triglycerides are unwanted and can lead to liver diseases. Since we did not measure the exact level of each composition, it cannot be concluded that which components are dominantly increased. Aside of this, based on the reference in the table 2, the increase of cholesterol is still in the normal category and therefore is too low to cause any damage to the liver or other organs. AV3 might have worked with controlling one or some components by increasing the level of them. Unlike our result, study of VFU Brno reported that there is no significance in cholesterol. It may be attributed by some reasons such as feed ingredients, age and biological state of rabbits.

Mahadevan et al., conducted a study on ginkgo biloba nuts and their extracts on cholesterol levels. They found that most of the concentration of cholesterol in serum was higher after administration. They claimed water soluble fractions might be responsible in the increase of cholesterol while lipid soluble fractions contributed the decrease of cholesterol in the liver (Madevan et al., 2007). It might probably the extracts ginkgo biloba in AV3 contains high amount of water soluble fractions. An insignificant increase of cholesterol level (LDL) after inclusion of ginkgo biloba extract was shown in Şener et al. experiment. However, the protective activity of ginkgo biloba cause significant decline in enhanced-LDL Hg-induced group of rats (Şener et al., 2007).

Lamant et al., proposed the mechanism of pharmacological actions on GBE might include stimulation of the discharge of EDRF/NO and PGI2, PAF (platelet activating Factor)-antagonism, antioxidant activity, trigger release of neurotransmitters and restriction of amine uptake. (Lamant et al.,1987).

GBE is obtained from the leaves and comprised of many flavonoid glycosides and terpenoids (ginkgolidae and bilobalide), while flavonoid is deemed to play a great role in antioxidant activity which greatly influence on the pharmacological effects. The protective functions of GBE against cell damage is commonly connected with scavenging activity of the flavonoids on reactive oxygen species (superoxide radical, peroxyl radical and nitric oxide). However, terpenoid contents may also contribute to free radical inhibition, so have cardioprotective activity. While Zhang et al., suggested the protective effect of GBE on liver injured cells may be attributed to the hindrance to lipid peroxidation and PAF (Zhang et al., 2004). These actions make GBE very beneficial in various disorders which oxidants are involved.

Many similar experiments have also evidenced the efficacy of GBP in alleviating the damage caused by oxidation, it worked by restoring the abnormal blood properties, this includes Şener et al., 2006; Şener et al., 2007; Yao et al., 2007; Panda and Naik, 2008.

There were also no significant results in growth performances, daily feed intake, FCR, and average daily gain between AV3 group and control group. It has shown that there was no influence of the addition AV3 0.20 ml/kg on those performances. The significantly improved kidney weight may correlate with the increase of total cholesterol level, especially triglycerides which bring fat in the blood.

There was a significant increase in carcass yield on AV3-fed group. The active substances MTE and GBE may enable the animals have better assimilation of protein in the gastrointestinal tract and lead to better conversion into the meat.

There are some possible reasons of these insignificant results. First, the dosage of AV3 might not be proper enough to give significant impact on the biochemical properties. Second, both milk thistle extracts (MTE) and ginkgo biloba extracts (GBE) only give significant effects if there were any abnormality of status, for example unhealthy or damaged organ functions.

We suggest that one of the main reasons of this insignificancy was the concentration of both MTE and GBE were too low. The amount of feed additive given to animals also depends highly on the species. The dosage 0.20 ml/kg of mixed MTE and GBE was not enough to give significant impact on rabbits performances.

Lutsenko et al., have shown there were some positive changes occurs when the chicks were fed by additional silymarin in a dose of 0.6 mg/kg in feed ration. These include the level of albumin and total protein. They claimed that the chicks suffered from Hypoproteinemia due to long feeding with an improper protein ratio of feed while silymarin treated group can normalize this deleterious impact. Silymarin may help in better feed intake to acquire more digestible protein, providing better assimilation in gastrointestinal tract and inducing the production of liver protein. (Lutsenko et al., 2008)

The other strong reason was because AV3 may act greatly only on animals which have possessed abnormality in the status. In the research on CCl4 induced Hepatotoxicity in albino rabbits, Jain et al., reported than silymarin could normalize not only the biochemical status, but also the feed intake, liver weight, and body weight. (Jain et al., 2013). In this experiment, there was no deleterious inducement that can be harmful for the rabbits. So, there were no any abnormality in the status that affect the growth, feeding activity, alterations in organs, and so on.

Our experiment started at 42-day old rabbits and ended when the rabbits had reached weights of 2600 grams. This weight was used as a measurement to sample collection due to it is an ideal weight to be slaughtered. Most of the rabbits in our experiments reached this weight before 85 days of age. Since we emphasize on fattening rabbits, we omit those rabbits which older than 85 days of age but have not reached the desired weight. Fattening rabbits usually reach the slaughtered weight before 85 days, more than that it is considered too slow and not productive. Since this limitation, the

rabbits were still young, the endogenous antioxidant systems were still fully functional to maintain their homeostasis and the organs were still intact. These reasons may also attribute to the insignificant results of biochemical properties in this experiment.

Oxidative stress is a pathogenetic mechanism leading to organ damage, occurred when there is a surplus of reactive species developed from oxygen or nitrogen. (Medina and Moreno-Otero, 2005). Apart from that, it can also happen due to shortage of antioxidant molecules.. Reactive oxygen species contribute to defects in carbohydrates, DNA, proteins, lipids, enzymes, and transporters. In normal organs, for example liver, either enzymatic or non-enzymatic antioxidants will give protection. In the normal state, antioxidants can cope with the deleterious oxidative agents in the balanced level. Aging is usually connected with the rise of oxidation. Aging can lead to an imbalance between antioxidant and oxidant which may cause many diseases. There are some age-dependent alteration, including reduction in organ mass, a decrease of mitochondria, bile duct formation, liver enlargement and degeneration. These changes may influence the biochemical properties in blood. If this experiment were using aged rabbits, some significant results would occur.

MTE contains bioflavonoids silymarin which represents up to 80% of MTE and comprised of more than 7 flavonolignans and one flavonoids, a taxifolin. Nik et al,. used Silymarin as feed additive of broiler feed. In concurrence with our results, there were many insignificant results of its impact on blood chemical properties such as total protein, glucose, albumin, globulin, AST and ALT in their experiment (Nik et al., 2014).

There are so many evidences indicating that the use MTE or GBE alone in healthy animals did not give any significant influences on many biochemical activities in healthy animals.

In 2008, the use of silymarin on healthy broilers in the experiment conducted by Lutensko et al. resulted in no difference in ALT and AST serum activity, but significant increases of total protein, calcium, phosphorous, albumin and globulin levels compared to the control ($P<0.05$) (Amiri Dumari et al., 2014)

Furthermore, in the study of J Kucharz and Kott on the effect of silymarin on hepatic functions in normal rats, silymarin could only increase total protein while there were no significant results of total bilirubin, alanine amino transferase and γ-glutamyl transpeptidase. In contrast, in damaged liver due to salicylic acid inducement, silymarin worked very well in normalizing the level of hepatic functions, even if it could not restore

them. Silymarin may act to limit the drug mechanism by attaching themselves to microsome membranes, this binding may reduce the susceptibility of the drugs (J Kucharz and Kott, 1994). Maryam et al reported that bilirubin, AST and ALT were not significantly changed by the administration of silymarin in rabbit blood but were restored after the abnormality due to oxidative stress inducement (Maryam et al., 2010). Many researches assume that silymarin are also capable of lessening liver damage by stabilizing and maintaining the integrity of cell membranes, therefore limiting the discharge enzymes through membranes (Oda and El-Ashmawy, 2012).

The combination of two plant extracts may give better results because each of them possesses unique capability of fighting against deleterious compounds. The combination of two or more plant extracts have been conducted in many researches. Similar results to our study, Shaarawy et al., reported that the combination of silymarin and garlic had given no significance results on ALP, ALT and AST (Shaarawy., 2009).

Panda and Naik have tried using different concentrations (100 mg/kg and 200 mg/kg) of ginkgo biloba phytosome (GBP). They found that both concentrations gave no impact on enzymes AST, LDH and CPK of normal liver rats. In contrast, Isoproterenol-induced elevated AST, LDH and CPK in rats could be restored by either the low or high concentration of GBP. They also claimed that the high (200 mg/kg) concentration gave more effect on ameliorating the impairment (Panda and Naik, 2008).

The efficacy of both MTE and GBE as antioxidant have been proven many times. However, again, the previous experiment was always conducted separately and never combined them together. Based on the result of our experiment, the concentration of non-antioxidant enzyme glutathione was increased after the addition of AV3, however, the increase was not significant. This result corresponds with the result of GSH by VFU Brno in the dams.

Reduced glutathione (GSH) was chosen as a measurement parameter of antioxidant because of its great function of protectors from deleterious oxidations. GSH plays a great role in the defence cell mechanism against deleterious oxidative stress in cytoplasm. It constitutes the major part of endogenous non-protein sulfhydryl pool. Reduced glutathione can form oxidized glutathione (GSSG) after stabilizing molecules (free radicals). The lack of electron giving reduced glutathione more reactivity results in joining each other. The increase of reduced glutathione indicated there were more antioxidants to scavenge the free

radicals. The amount of GSH varies considerably depending on species, location in the body, and methods to obtain it.

The response of MTE or GBE to the increase of GSH may be influenced also by several factors. Ramadan et al, reported that the efficacy of silymarin in improving depleted GSH given intravenously was better than oral treatment (Ramadan et al., 2002).

Reduced glutathione was collected from liver, it was because liver contains the highest level of reduced glutathione, up to 10 mmol/l GSH, while the concentration in plasma is only 2-20 µmol/l. The high content of glutathione in liver is understandable since liver is the major site for metabolism, reactive oxygen species are repeatedly created in response to the normal and drug metabolism. Hepatocytes are cells which accounted for up to 85% of liver mass, the overabundance of reactive oxygen species (ROS) produced in this area may lead to cell death by impairing DNA, lipids, proteins and carbohydrates (Habib-ur-Rehman et al., 2009). So, the high level of glutathione is needed to stabilize liver functions. Liver defensive mechanism starts to be unstable if GSH level is less than 20% of the normal state (Ilyas et al., 2011).

Plenty of prior experiments on the impact of MTE or GBE on the improvement of GSH have been done. This result of GSH was in conjunction with Ahmad et al. in which silymarin could improve and restore depleted GSH due to oxidative stress inducement. The mechanism of actions of silymarin in combating oxidative stress is still poorly documented. However, the restoration due to silymarin inclusion may be caused by the increase of glutathione or the decrease of oxidative stress. The decrease of oxidative stress may be from the ability of silymarin in enhancing enzymatic antioxidants (SOD, CAT) (Rasool et al., 2014). The restoration capability of silymarin on GSH after severe oxidative damage has also been mentioned in earlier reports, include Maryam et al., 2010; Hakova et al., 1996; Oda and El-Ashmawy, 2012.

Similar effect of GBE on the improvement of GSH has also been concluded by many researches. GBE was also able to enhance the level of antioxidant enzymes, such as glutathione peroxidase and superoxidase dismutase. (Janssens et al., 2000)

Although in this research the effect of AV3 on GSH metabolism were not observed, some other related studies evaluate it. The evaluation was based on the activity of GSH enzymes such as glutathione peroxidase (GPx) and glutathione reductase (GR). Glutathione peroxidase is an enzyme catalyzing in the neutralization of peroxides compounds leading to GSSG forms. GSH will then be reformed by reduction activity of

GR with NADPH as the electron donor. These enzymatic glutathione enable the body system to maintain the endogenous antioxidant pool. Sylimarin is believed to be able to restore and maintain the GSH homeostasis in the system (Maryam et al., 2010) due to its capacity in improving enzymes associated with glutathione.

In Ahmad et al. experiment, silymarin could elevate the depleted GR activity caused by the inducement of oxidative stress, which is vital to synthesis more GSH. Ahmad also claimed that pretreatment with silymarin could reduce the level of GPx and they deemed the reduction was due to less oxidative stress exposed. In this research silymarin were proved to be able to protect body system from oxidative stress by reducing the oxidative stress as well as to support the creation of GSH (Ahmad et al., 2013).

Understanding the response of GBE in healthy liver is almost absence, it is believed that the significant improvement of GSH level in our results was due to the action of MTE.

The increase of GSH was not significant compared to the control. The best possible reason is the weak responds of AV3 to healthy animals. Many studies have been conducted to examine both of the extracts on the GSH level of animals. As far as we concern, either MTE or GBE has great potential in ameliorating the GSH level of damaged organs of animals. However, none of them have significant impacts on the GSH level of healthy animals. The dosage of AV3 might not give strong influence on the alteration of GSH since many studies have reported high concentrations of both MTE or GBE could not lead to an increase of GSH in healthy animals. In 2005, Yao et al., ran an experiment that demonstrated the addition of 96 mg/kg of GBE as feed supplementation in healthy rats resulted in an insignificant increase in liver, heart, kidney as well as the testes. Interestingly, they could lessen the significant drop of GSH better than a lower concentration of 48 mg/kg in intoxicated rats (Yao et al., 2005). Similarly, in the study of Þener et al, the inclusion of GBE in the dietary feed of hepatic fibrosis rats could restore depleted GSH. However, the inclusion of GBE in the feed of healthy rats did not show any significant difference. These results in an agreement with other studies, include Panda and Naik, 2008; Panda and Naik 2009; Şener et al., 2006.

Low (100 mg/kg) and high (200 mg/kg) of GBP were used by Panda and Naik in order to obtain the best dosage to enhance or restore GSH. Either the low or high concentrations of GBP did not significantly alter the GSH level of healthy animals. Surprisingly, the dose of 200 mg/kg GBP could significantly alleviate the depleted

isoproterenol-induced GSH level in rats, whereas the dose of 100 mg/kg could not any give significant results (Panda and Naik, 2008)

The insignificant result of GSH in this experiment corresponds with Oda and El-Ashmawy result. In his experiment 200mg/kg/bwt/day of silymarin solution was administered. The result was silymarin could not give significant results on GSH in healthy rabbits. However, significant results were achieved when it was administered to nephro-hepatotoxicity rats.

In spite of the action of GSH as an electron donor, GSH can also inhibit metal such as mercury. GSH can bind with the metal to prevent them from connecting with cellular proteins. In previous study, depleted GSH due to bonding with mercury can be ameliorated by the pretreatment with silymarin (Oda and El-Ashmawy, 2012).

MTE were also reported by Chand et al. that it also strengthen immune system not only due to its powerful antioxidant, free radical scavenging activity and glutathione formation but it is also capable of improving immune cells against immunosuppresion directly. In this result, however, AV3 also did not show promising results in improving immunity as showing an increase of mortality percentage. Nik et al. administered milk thistle and thyme seeds (alone and combined) to investigate the changes of blood chemical, lipid profile and immune response in broiler chicks. Some significant changes occurred in blood chemical and lipid profile, but not for the immune response (antibody titers viruses, heterophil and lymphocyte ratio and immunoglobulins). They mentioned that the insignificant results on immune-related parameters may be caused by the low dosage of additives either milk thistle seeds or thyme seeds (Nik et al., 2014). Our suggestion is in line with Nik's, so it is understandable that low level of AV3 supplemented in this research could give significant impact on immunity. Unfortunately, this research was more focused on biochemical properties in blood so, the details about immunity parameters were not observed. It is necessary to carry out further analysis on the effects of AV3 on immune-related parameters.

In this experiment there were significant results of the repetitions. This may be because the whole repetitions were not done at the same time, instead they were done in different time frame. These time frames involve the change of the seasons that may affect on the well-being of rabbits.

8 Conclusion

In the recommended dosage AV3 do not give significant changes in most of blood biochemical properties, growth parameters and reduced glutathione in liver of fattening rabbits. Besides cholesterol, AV3 did not give any influences on blood biochemistry of fattening rabbits. Cholesterol level was significantly enhanced by 38.6% in AV3 group compared to the control. There is no different concentration between AV3 group and the control. AV3 also did not show promising results in growth performances besides in better carcass yield performance. It is hard to measure the optimum dosage of MTE and GBE in AV3 to give significant values. Higher concentration of AV3 active compounds seems necessary if AV3 is supplemented on feedstuff of rabbits. It can be concluded that the dose 0.20 ml/kg AV3 seems neither to have any positive nor negative impacts on healthy fattening rabbits. However, from the economic perspective, the use of AV3 may seem not so cost-effective. There is a need in further research to obtain the best concentration that really gives benefits to fattening rabbits. It is also necessary to carry out experiment on the action of AV3 for sick or injured animals in order to know the protective and restoration effects of AV3.

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