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In-vitro Re-evaluation of Antioxidant activity by 2, 2-Diphenyl-1picrylhydrazyl Free Radical (DPPH) Assay in Medicinal Plants of Andhra Pradesh, India

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Abstract

Medicinal plants are a major source of raw materials on the globe from ancient to present century for the traditional system like Ayurveda, Siddha and Unani. Even the modern system of medicine has more than 25 percent of drugs in use which are either plant based or plant products. In day to day life, particularly in India people are suffering from menacing health diseases from common cold to amnesia and poisonous snake bites. These diseases can be treated by using some herbal extracts from plants which has antioxidant activity. Medicinal plants like *Azadirachta indica*, *Ocimum sanctum*, *Lawsonia intermis*, *Murraya koenigii*, *Curcuma longa* and *Cuminum cymium* belonging to different families play a vital role in day to day usage of different indigenous communities due to its sacred and medicinal value. Recently there has been an upsurge of interest in the therapeutic potential of medicinal plants as antioxidants. In the course of finding potential antioxidant from plant source, six medicinal tree species belonging to different families has been selected. Leaves were dried and extracted with methanol solvent systems. Antioxidant activity using 1, 1-diphenyl-2-picryl hydrazyl radical scavenging assay, of six extracts from six genus of different families are reported and a comparison of the free radical scavenging ability of the extracts is emphasized. The highest percentage of 1,1-diphenyl-2-picryl hydrazyl free radical scavenging activity is found in Azadirachta *indica (*81.8% of inhibition) which shows antimicrobial, anti inflammatory and anticancerous properties where Ascorbic acid (68.5 µg/ml) is taken as standard for comparison.

1. Introduction

India has one of the richest plant medical cultures on the globe. The term antioxidant in the beginning was used to refer indicatively to a chemical that averted the consumption of oxygen (**Shahidi and Naczk, 2004; Tachakittirungrod et al., 2007**). Antioxidant activity could be measured simply by placing the fat in a closed container with oxygen and measuring the rate of oxygen consumption (**Tomaino et al., 2005**). The recent growth in knowledge of free radicals and reactive oxygen species (ROS) in biology (**Bravo, 1998; Martinez-Valverde et al., 2000**) is producing a medical rebellion that promises a new age of health. Free radicals and related species have attracted a great deal of attention in recent years. They are mainly extracted from oxygen, ROS and nitrogen (reactive nitrogen species/RNS), and are generated in our body ,various endogenous systems, exposure to different physico chemical conditions or patho physiological states However ROS levels are elevated by exogenous factors such are pollution, smoke, radiation, pesticide, drug consumption. In the case of disturbed balance between formation of free radicals and antioxidant shielding (**Roginsky and Lissi, 2005**), there is an oxidative stress which can leads to development of various diseases (**Arganosa et al., 1998**), including cardiovascular diseases , diabetes, cancer, Alzheimer's diseases, retinal degeneration, ischemic dementia, neurodegenerative disorders aging, trauma, stroke, and infection. ROS are entire class of highly reactive molecules derived from the metabolism of oxygen. ROS, including superoxide radicals, hydroxyl radicals, and hydrogen peroxide, are often generated as byproducts of biological reactions or from exogenous factors. In addition to antioxidant enzymes, non-enzymatic molecules, including thio-redoxin, thiols, and disulfide-bonding plays an important role in antioxidant shielding systems. Antioxidant based drugs/formulations for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's diseases, and cancer have appeared during the last three decades.

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Antioxidants shielding system (**Farombi et al., 2000; Jaffel et al., 2011**), act as radical scavenger, hydrogen donors, electron donor, peroxide decomposer, single oxygen quencher, enzyme inhibitor, synergist, and metal-chelating agents. To provide maximum intracellular protection these scavengers are strategically compartmentalized thought the cell. According to some plant secondary products are of particular interest are Plant phenolics, polyphenolic, alkaloids, non-proteins amino acids,

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isothiocyanate, indoles phytosterols, carotenoids, chlorophyll derivatives.

1.1 Antioxidant Based on Defense Mechanism: These are of three types:

1. Prevention antioxidants- These suppress the free radical formation.

Ex. Enzymes such as peroxidase, catalase, lactoferrin, carotenoids, etc.

2. Radical scavenging antioxidants – These suppress the chain initiation reaction. Ex. Vitamin-C & Carotenoids.
3. Enzyme inhibitor antioxidants – These induce production and reaction of free radicals and the transport of appropriate antioxidants to appropriate active site.

1.2 Formation of Free radicals

Oxygen radicals **(Gilbert DL, 1981)** are generated from the triplet state oxygen by excitation or reduction. "Superoxide theory of oxygen toxicity" states that oxygen is toxic because it can form superoxide radical, peroxynitrite and hydroxyl radicals which can initiate autoxidation, polymerization and fragmentation.

Before we understand the working, it is necessary to have a brief idea about free radicals (**Roginsky and Lissi, 2005**). During a chemical reaction (oxidation), one reactant loses an electron and is called oxidant or free radical, while the other gains an electron. In living organisms oxygen in unstable from is the most common free radical. This is called Reactive Oxygen Species and is generated during various metabolic activities. Contaminants as well as normal metabolism of cell can change molecule into a free radical. The examples of ROS are OH, O_2, H_2O_2, O_3, HOCI, RO_2, and RO.

Any molecule can become a free radical by either losing or gaining an electron. Once initiated these free radicals get involved in chain reaction with stable types. The compounds thus formed have longer stability and in body and increase the potential for cellular damage. Free radicals damage the cell at the site of their operation causing serious disorders. Plaque may accumulate in arteries on oxidation. LDL Cholesterol functions as free radical and damages the free artery lining. It hampers the blood circulation which may lead to heart attack.

As oxidative stress might be an important part of many human diseases, the use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases. Free radicals can adversely alter lipids, proteins and DNA and have been implicated in aging and a number of human diseases. Lipids are highly prone to free radical damage resulting in lipid peroxidation that can lead to adverse alterations. Free radical damage to protein can result in loss of enzyme activity. Damage caused to DNA, can result in mutagenesis and carcinogenesis. Redox signaling is a major area of free radical research that is attracting attention. ROS are an entire class of highly reactive molecules derived from the metabolism of oxygen. ROS, inducing superoxide radicals, hydroxyl radicals, and hydrogen peroxide, are often generated as byproducts of biological reactions or from exogenous factors.

2. Material and methods

2.1 Materials

2.1.1 Chemicals and Reagents

The chemicals and reagents obtained from the biochemistry laboratory for calculating the in-vitro antioxidant activity of six medicinal plants extracts are:

1, 1-Diphenyle-2-picryl hydrazyl (DPPH), Ascorbic acid, methanol, Sodium carbonate (Na2CO3), phosphate buffer (pH-6.6) and distilled water.

2.1.2 Apparatus

Absorbance spectrophotometery was carried out using a UV-VIS spectrophotometer. Wavelength scans and absorbance measurements were in 1ml quartz cells of 1cm path length. Beakers, test tubes, measuring cylinders, analytical balance, micro pipettes, motor and pestle, glass rods, centrifuge, pipettes, micro-tips, conical flasks, centrifuge tubes, incubator, hot air oven, test tube stand, magnetic stirrer, filter papers, foils, marker and shaker.

2.1.3 Plant materials

The plant leaves of *Azadirachta indica, Ocimum sanctum, Lawsonia intermis, Murraya koenigii,* the rhizome of *Curcuma longa* and the seeds of *Cuminum cyminum* are the medicinal plant materials collected from different regions for calculating the antioxidant activity by free radical scavenging assay.

Botanical Name : *Ocimum sanctum*
Family : *Lamiaceae*
Vernacular Name : Tulsi are Manjari, Krishna Tulsi, Trittavu, Tulshi and Thulsi
Part used : Leaves

Habitat: Tulsi is a heavy branched having hair all over. It attains the height of about 75 – 90 cm. It has round oval shaped leaves which are up to 5 cm long. The leaves are 2- 4 cm in length. Its seeds are flat. Its flowers are purple – creamish in colour. The Tulsi with the green leaves is called the Shri Tulsi and one with the reddish leaves is called the Krishna Tulsi. Its seeds are yellow to reddish in colour. Leaves of Tulsi contain very essential oil.

Medicinal properties: The fresh leaves of Tulsi are taken by the millions of people every day. The leaves gives relief in cold, fever, bronchitis and cough. "Modern scientific research offers impressive evidence that Tulsi reduces stress, enhances stamina, relieves inflammation, lowers cholesterol, eliminates toxins, protects against radiation, prevents gastric ulcers, lowers fevers, improves digestion and provides a rich supply of antioxidants and other nutrients. Tulsi is especially effective in supporting the heart, blood vessels, liver and lungs and also regulates blood pressure and blood sugar.

Botanical Name : *Azadirachta indica*
Family : *Maliaceae*
Vernacular Name : It is popularly known as the miracle tree. Nimba and Neem
Part used : Leaves

Habitat: Neem tree is found throughout India. It is a popular village tree. Neem tree can easily be grown in the dry, stony, shallow and clayey soils.

Medicinal properties: Neem leaves to cure skin diseases such as boils, ulcers, eczema, and ring worm, anti inflammatory, antipyretic and hypoglycemic **(Porter WL, 1986)** and also exhibits antimicrobial and anticancerous properties. Neem leaves are traditionally being used as curative against certain fungal and bacterial diseases. However, evaluation of its antiviral properties is limited to few viruses viz. Measles, Chicken pox, HSV and HIV.

Botanical Name : *Lawsonia intermis*
Family : *Lythraceae*
Vernacular name : *Henna*
Part used : *Leaves*

Habitat: Henna is a tall shrub or small tree, 2.6 m high.

Medicinal properties: Henna also acts as an anti-fungal and a preservative for leather and cloth.

Botanical Name *: Murraya koenigii*
Family : *Sprengel Rutaceae*
Vernacular Name : *Curry leaves*

Part used : *Leaves*
Habitat: Curry leaf is a traditional spice used in south India for all the curry preparations. The plant *Murraya koenigii* (L.) *Spreng,* belonging to the family *Rutaceae* is native to India and distributed in most of Southern Asia.
Medicinal properties: The leaves increase digestive secretions and relieve nausea, indigestion, and vomiting. This species is known to possess anti inflammatory, antidysenteric, antioxidant, antidiabetic and diverse pharmacological properties.
Botanical Name : *Curcuma longa*
Family : *Zingiberaceae*
Vernacular Name : *Turmeric, Yellow zinger, Curcuma, haldi, pasupu*
Part used : *Rhizome*
Habitat: Turmeric is the rhizome or underground stem of a ginger-like plant. It is usually available ground, as a bright yellow, fine powder. The main rhizome measures 2.5 - 7 cm (1" – 3 ") in length with a diameter of 2.5 cm (1"), with smaller tubers branching off.
Medicinal properties: Turmeric is currently being investigated for possible benefits in **Alzheimer's disease**, **cancer**, arthritis, and other clinical disorders.
Botanical Name : *Cuminum cyminum*
Vernacular Name : *Cumin, Jeera*
Family : *Apiaceae*
Part used : *Seeds*
Habitat: Cultivation of cumin requires a long, hot summer of 3–4 months, with daytime temperatures around 30 °C (86 °F); it is drought-tolerant, and is mostly grown in Mediterranean climates. It is grown from seed, sown in spring, and needs fertile, well-drained soil.
Medicinal properties: Jeera acts as an anti-obesity, anti-inflammatory, blood purifier, diuretic, glactogauge (that enhances milk engendering during lactation) and uterine stimulant medicine. Jeera is also a very good medicine for mucous diarrhoea and non-specific colitis and is used in combination with other medicines to cure the irritable bowel syndrome.

2.2 Methods

2.2.1 Preparation of Extracts
Fresh leaves of Plants were cut into small pieces, dried in the sun for seven days. And finally in an oven below 60 °C .The dried plant materials (1 kg) was ground into fine powder using motor and pestle and then exhaustively extracted with methanol. The extract was concentrated to a dark greenish residue. This crude extract was used for further investigation for potential antioxidant properties.

2.2.2 Antioxidant Assay
The **antioxidant activity** of Plant extracts were determined by **in- *vitro*** methods (**Zou et al., 2004**). Such as:

- The DPPH free radical scavenging assay

The assay (**Proestos et al., 2006**) was carried out in triplicates and average values were considered.

2.2.3 DPPH radical scavenging activity
The antioxidant activity of the methanolic extracts was determined on the basis of their scavenging activity of the stable 1, 1-diphenyl-2-picryl hydrazyl (DPPH) free radical (**Hanato et al., 1988**). DPPH is a stable free radical containing an odd electron in its structure and utilized for detection of the radical scavenging in chemical analysis. Due to the presence of an odd electron it gives a strong absorption maximum at 517 nm. As this electron becomes paired off in the presence of a hydrogen donor, i.e. a free radical scavenging antioxidant, the absorption strength is decreased, and the resulting decolorization is stoichiometric with respect to the number of electrons captured as shown in Fig.1. One ml of each solution of different concentrations (10-100µg/ml) of the extracts was added 3ml of 0.004% methanolic DPPH free radical solution and reaction mixture was shaken vigorously. These solution mixtures were kept in dark for about 30 minutes and the absorbance of the preparations were measured at 517 nm using a UV spectrophotometer which was compared with the corresponding absorbance of the standard ascorbic acid of different concentrations (10-100 µg/ml). Then the % of inhibition was calculated by the following equation:
% DPPH radical scavenging activity = [(Absorbance of control – Absorbance of test sample) / (Absorbance of control)] × 100.

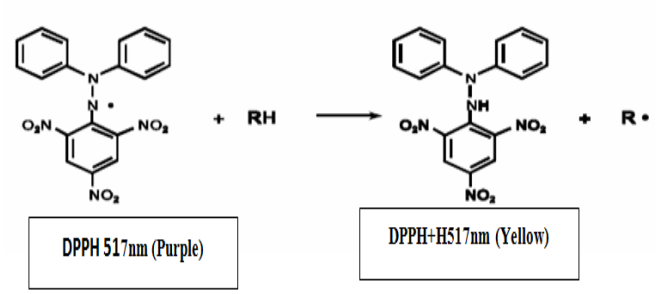

Figure 1. DPPH radical scavenging activity.

3. Results and discussion

Free radicals are inextrabically linked to various disorders, causing different diseases (**Cook and Samman, 1996**). Many plant products have exact antioxidant effect by quenching various free radicals and the singlet form of molecular oxygen**,** (**Liu et al., 2007; Salem et al., 2011**).Various methods have been proposed to evaluate antioxidant characteristics and to explain antioxidant function of plant products. Of those antioxidant activity, different types of free radical scavenging activity is most commonly used for the evaluation of the total antioxidant behavior of extracts.

3.1 DPPH Radical Scavenging Activity
DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule (**Kumaran and Karunakaran, 2007**). Hence DPPH is usually used as substrate to evaluate the antioxidant activity (**Molyneux, 2004**). The strength of this scavenging activity of methanol extract and standard on DPPH radical revealed the scavenging activity in terms of percentage of inhibition. The following Table 1. shows the results of DPPH radical scavenging activity of selected six medicinal plants and ascorbic acid as standard. The Figure 2. shows the DPPH radical scavenging activity of Ascorbic acid, Azadirachta indica, Ocimum sanctum, Lawsonia intermis, Murraya koenigii, Curcuma longa, and Cuminum cyminum.

Table 1. Antioxidant activities of medicinal plants in Methanol.

Concentration of plant extracts (µg/ml)	% of inhibition						
	Ascorbic acid	Azadirachta indica	Ocimum sanctum	Lawsonia intermis	Murraya koenigii	Curcuma longa	Cuminum cyminum
10	58.3	40	50	54.5	28.5	37.5	40
20	61.5	50	50	57	55.5	58.3	57.1
40	64.2	50	57.1	57.1	58.3	64.2	64.2
60	66.6	66.6	62.5	58.3	55.5	61.5	66.6
80	68.5	66.6	62.5	58.3	55.5	75.0	68.7
100	68.5	81.8	66.5	66.6	63.6	75.0	75.0

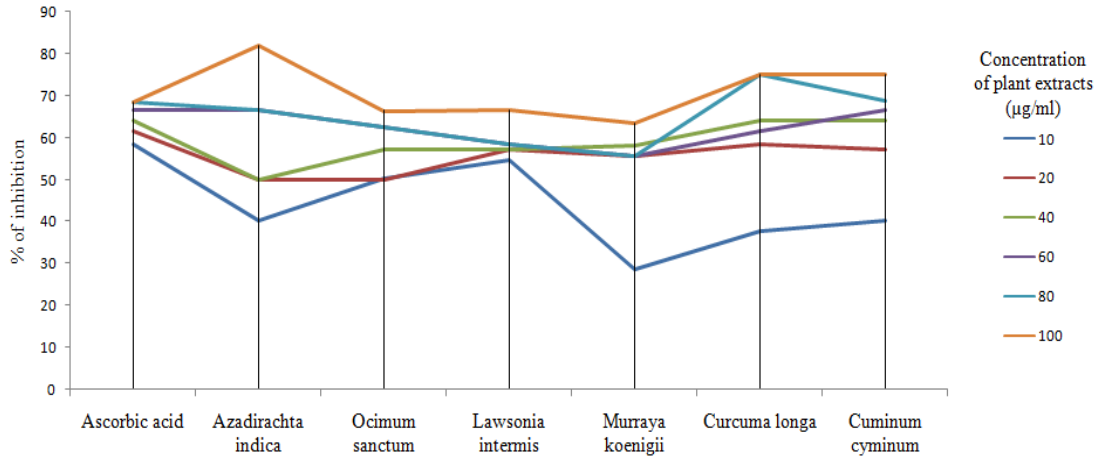

Figure 2. DPPH radical scavenging activity of Ascorbic acid, Azadirachta indica, Ocimum sanctum, Lawsonia intermis, Murraya koenigii, Curcuma longa, and Cuminum cyminum

The percentage of inhibition shown by Azadirachta indica, Curcuma longa and Cuminum cyminum is 81.8%, 75% and 75% respectively (**Wangensteen et al., 2004**). Which is greater than the percentage of inhibition of standard i.e. ascorbic acid - 68.5 And the methanolic extracts of Ocimum sanctum, Lawsonia intermis and Murraya koenigii has also shown significant activity i.e. 66.5%, 66.6% and 63.6% respectively, as shown in Table 2 as well as shown in Figure 3.

Table 2. Percentage of DPPH Radical Scavenging Activity

S.No.	Plant Species	Plant part used	%of inhibition
1	*Azadirachta indica*	Leaves	81.8
2	*Curcuma longa*	Rhizome	75
3	*Cuminum cyminum*	seeds	75
4	*Ocimum sanctum*	Leaves	66.5
5	*Lawsonia intermis*	Leaves	66.6
6	*Murraya koenigii*	Leaves	63.6

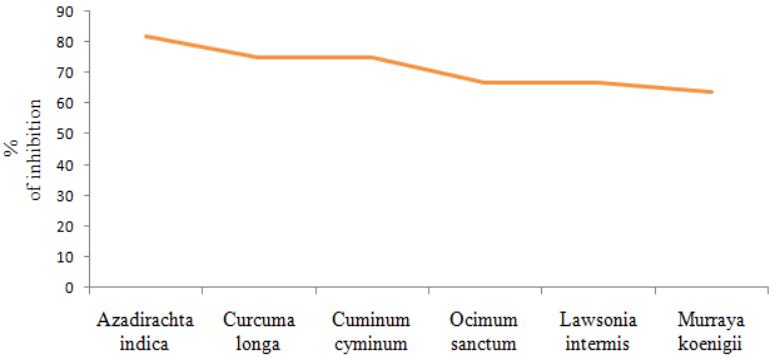

Figure 3. DPPH radical scavenging activity of selected six medicinal plants

Based on the data obtained from the study, methanolic extracts of these plants exhibited high scavenging activity and can minimize or reduce free radical damaging occurred in the human body (**Sriti et al., 2011**).

4. Conclusions

In conclusion, the results of the present study that the methanolic extracts of *Azadirachta indica, Curcuma longa , Cuminum cyminum, Ocimum sanctum, Lawsonia intermis and Murraya koenigii* exhibits significant antioxidant activity through the scavenging of different free radicals which participate in various pathophysiology of diseases. Standardized aqueous methanolic extracts from the selected

leaves and seeds having different target radicals, such as superoxide radical, nitric oxide, and peroxidative decomposition of phospholipids, were prepared and screened by *In-vitro* assays. Fresh leaves of Plants were cut into small pieces, dried in the sun for seven days. And finally in an oven below 60 °C .The dried plant materials (1 kg) was ground into fine powder using motor and pestle and then exhaustively extracted with methanol. The extract was concentrated to a dark greenish residue. This crude extract was used for further investigation for potential antioxidant properties. These extracts were tested for, DPPH free radical scavenging activity correlated with antioxidant capacity (**Cheung et al., 2003**). The highest percentage of DPPH free radical scavenging activity is found in *Azadirachta indica(*81.8 µg/ml % of inhibition) which shows antimicrobial, anti inflammatory and anticancerous properties where Ascorbic acid (68.5 µg/ml) is taken as standard for comparison. Overall, the plant extracts is a source of natural antioxidants that can be important in disease prevention, health preservation and promotion of longevity of life. The antioxidants is important in industrial processes, such as the prevention of metal corrosion, the vulcanization of rubber, and the polymerization of fuels in the fouling of internal combustion engines.

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The *In Vitro* Effect of Kanamycin on the Behaviour of Bovine Spermatozoa

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Abstract

The use of antibiotics is a common part of animal biotechnologies. Especially, the use of antibiotics in semen extenders is necessary. However, the effect of antibiotics on the spermatozoa structure and function is still not completely examined. Therefore, the aim of our study was to investigate the effect of kanamycin on bovine spermatozoa at concentrations of 80 and 160 µg/mL during the 24 h *in vitro* cultivation. Semen samples were collected from clinically healthy Holstein-Friesian bulls. At times of 0, 2 and 24 h the motility assessment, mitochondrial activity, acrosomal and membrane integrity evaluation were performed. The sperm motility was measured using the Computer-assisted sperm analysis (CASA). Mitochondrial activity was evaluated through the Mitochondrial Toxicity Test (MTT). The acrosomal status was determined using the fast green/rose bengal staining on slides. Similarly, the membrane integrity was analysed using the eosin-nigrosin staining. Our results revealed the dose- and time-dependent effect of kanamycin under the *in vitro* conditions. In conclusion, the selected concentrations of kanamycin may have adverse effects on the motility, mitochondrial activity, acrosomal and membrane integrity during semen processing. Considering the relatively low concentrations used, we do not recommend to use kanamycin as a supplement in bovine semen extenders.

1. Introduction

The collection of bovine semen samples is not a sterile process. Even thorough sanitary precautions cannot prevent the potential microbial load in semen. Not only during semen collection, but also during processing and storage of semen the sample may be contaminated. Even freezing is not an obstacle to the bacterial survival, colonization of semen sample and an outbreak of infection disease inside the recipient's uterus **(Paray *et al.*, 2018)**. Therefore, antibiotic treatment in semen storage media is necessary. However, the use of an optimal concentration of antibiotics is as important as the choice of antibiotic supplementation. The same concentration of the same antibiotic in the semen extender may, depending on the species, improve or deteriorate spermatozoa quality **(Morell and Walgren,2014)**.

Aminoglycosides are popularly used as supplements of semen extenders **(Morrell and Wallgren, 2014; Khaki, 2015)** due to their selective mechanism of action. Kanamycin (KAN) irreversibly binds to the bacterial 16s rRNA, causing misalignment of the amino acids during proteosyntesis leading to the breakdown of the polysomes into non-functional monosomes **(Wieninger *et al.*, 2011)**. Nowadays, various types of antibiotics or thier combinations are used to manage bacteriocenosis in ejaculates and potential risk of harmful effects of bacteria. Previous study recommended the dose of 80 µg/mL of KAN in semen extenders **(Di Iorio *et al.*, 2014)**. However, there are no studies about the effect of KAN on spermatozoa during their *in vitro* cultivation **(Stoianov, 1987)**. Therefore, the aim of our study was to evaluate the effect of the recommended concentration (80 µg/mL) and double concentration (160 µg/mL) of KAN on the motility, mitochondrial activity, membrane and acrosomal integrity of bovine spermatozoa during 0, 2 and 24 h.

2. Material and methods

2.1 Sample collection and processing

Semen samples (n=10) were collected from clinically healthy Holstein-Fiesian breeding bulls (Slovak Biological Services, a.s., Lužianky, Nitra, Slovakia). Each sample was subjected to the quality criteria depending on the breed. After collection, the semen samples were transported to the laboratory within 15 min. in constant temperature. Samples were diluted in physiological saline solution (PS; sodium chloride 0.9% w/v, Bieffe Medital, Grosotto, Italia) in a dilution ratio of 1:40. Kanamycin (KAN; Kanamycin sulfate, Sigma-Aldrich, St. Louis, USA) dissolved in PS was added to the semen samples at final concentrations of 80 and 160 µg/mL. The control group (Ctrl) consisted of PS and spermatozoa. Semen samples were incubated during 24 hours at room temperature (20-22°C). At times of 0, 2 and 24 h, motility assessment, mitochondrial activity, membrane and acrosomal integrity were analysed.

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2.2 Motility assessment

Basic analysis of the sperm quality, motility assessment, was carried out using the Computer-assisted semen analysis (CASA; Version 14.0 TOX IVOS II.; Hamilton-Thorne Biosciences, Beverly, MA, USA). Ten µL of each sample were placed into the Makler counting chamber (depth 10 µm, 37°C; Sefi Medical Instruments, Haifa, Israel) and subjected to sperm motility (MOT) analysis. Only spermatozoa moving at least 5 µm/s is considered to be motile. The percentage of motile spermatozoa was evaluated at a minimum of 300 cells in each sample (**Tvrdá et al., 2016**).

2.3 Mitochondrial activity assessment

Mitochondrial metabolic activity was analysed using Mitochondrial Toxicity Test (MTT). This colorimetric method is based on enzymatic conversion of a yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to purple formazan particles by succinate dehydrogenase of intact mitochondria within living cells. Twenty µL of tetrazolium salt (Sigma-Aldrich, St. Louis, USA) dissolved in PBS (Dulbecco's Phosphate Buffer Saline without calcium chloride and magnesium chloride; Sigma-Aldrich, St. Louis, USA) to concentration of 5 mg/mL were added to each sample and incubated during 1 h at 37°C. Subsequently, formazan crystals were dissolved using 80 µL of isopropanol (propan-2-ol; Centralchem, Bratislava, Slovakia). Optical density was measured using the Glomax Multi+ (Promega Corporation, Madison, WI, USA) at a wavelength of 570 nm against 620 nm as reference. The results are expressed as percentage of the Ctrl group set to 100% (**Tvrdá et al., 2016**).

2.4 The sperm membrane integrity

The standard eosin-nigrosin staining protocol was performed to evaluate the functional maintenance of the sperm membrane. The assay is based on the ability of eosin to penetrate into the cells and to distinguish non-viable cells from viable. Five µL of each sample were placed on a glass slide and immediately mixed with 10 µL 5% eosin (Sigma-Aldrich, USA), followed by 10 µL 10% nigrosin (Sigma-Aldrich, USA). The mixture was smeared on a glass slide and left to air-dry. Using the bright-field microscopy (1000 ×; minimum of 200 cells in each sample were evaluated based on a ratio of red heads (dead)/white heads (live). The results are expressed as a percentage of live spermatozoa (**Agarwal et al., 2016**).

2.5 The acrosomal status

Acrosomal integrity of sperm cells was evaluated according to staining protocol by **Pope *et al.* (1991)**. The staining solution consisted of 1% fast green, 1% rose bengal (both Sigma-Aldrich, USA) and 40% ethanol (Centralchem, Slovakia) dissolved in 0.1M citric acid–0.2M disodium phosphate buffer (Sigma-Aldrich, USA). Twenty µL of each sample was mixed with 20 µL of staining solution and incubated during 70 s at room temperature (20-22°C). Ten µL of the mixture was smeared on a glass slide and left to air-dry. The presence or absence of acrosome was performed by one observer using the bright-field microscopy (1000 ×). Minimum of 200 cells in each sample were counted. The results are expressed as a percentage of sperm with intact acrosome.

2.6 Statistical analysis

The GraphPad Prism program (Version 6.0 for MS Windows; GraphPad Software, www.graphpad.com) was used for statistical evaluation. All data are expressed as the arithmetic mean (AM) ± standard error of mean (SEM). One-way ANOVA with following Dunnett's test was used for specific statistical evaluations. The significance level was set at *** (P<0.001), ** (P<0.01) and * (P<0.05).

3. Results

Two concentrations of kanamycin were used to reveal the effect of kanamycin on the motility, mitochondrial activity, membrane and acrosomal integrity of spermatozoa. The CASA assessment (Figure 1) revealed already at initial time a significant decrease (P<0.01) in the group treated with 160 µg/mL of KAN when compared to the control group. Only a slight decrease was observed in the group treated with 80 µg/mL. After 2 h, spermatozoa motility significantly decreased in both experimental groups (P<0.01 in case of 80 µg/mL; P<0.001 in case of 160 µg/mL). Similar results were obtained after 24 h. When compared to Ctrl, spermatozoa motility decreased by treating 80 µg/mL (P<0.05) as well as 160 µg/mL (P<0.001) of KAN.

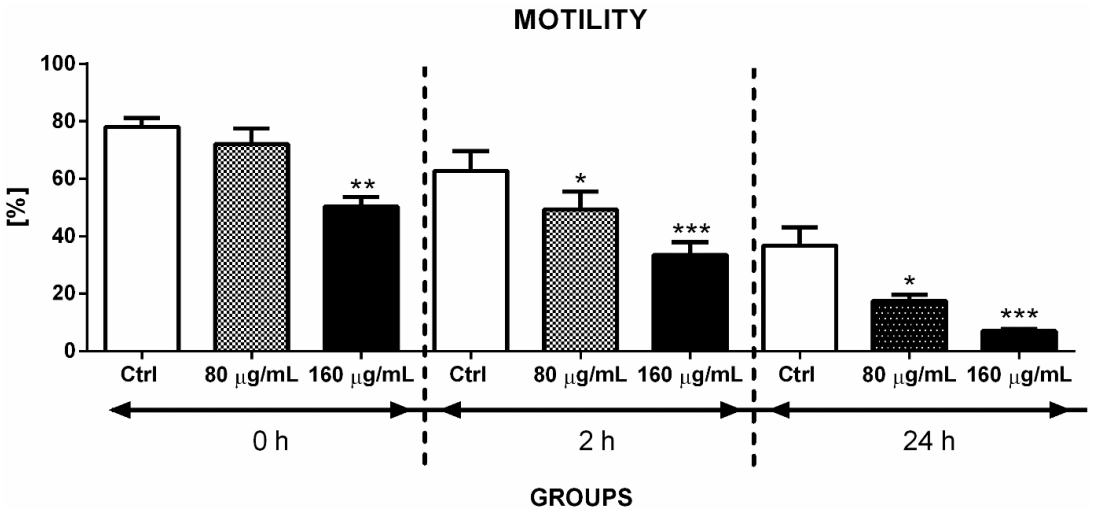

Figure 1. The development of spermatozoa motility in the groups treated with 0 (Ctrl), 80 and 160 µg/mL during 24 h storage of *in vitro* culture. The results are expressed as AM ± SEM. The level of significance was set at *** (P<0.001), ** (P<0.01) and * (P<0.05).

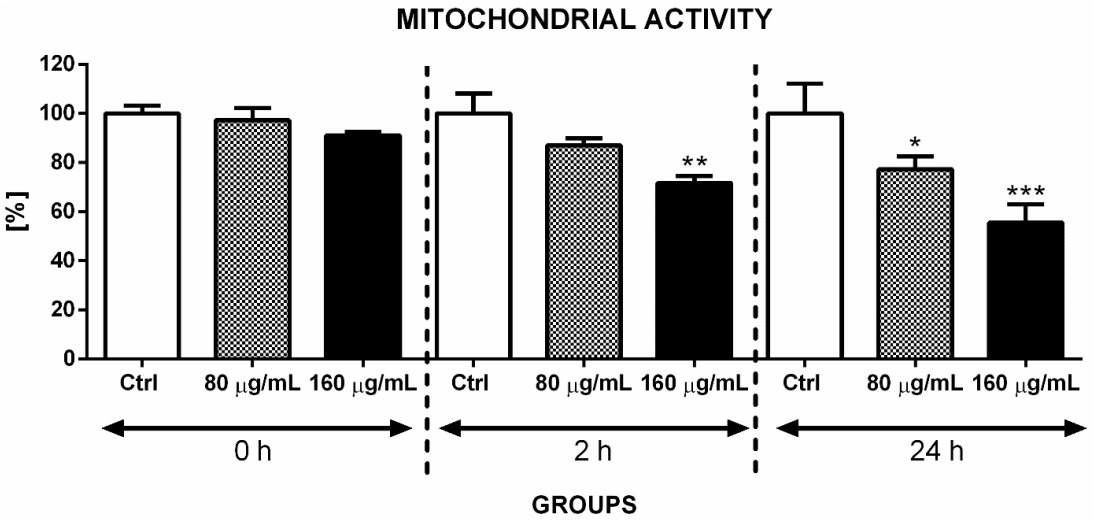

Figure 2. The development of mitochondrial activity of spermatozoa in the groups treated with 0 (Ctrl), 80 and 160 μg/mL during 24 h storage of *in vitro* culture. The results are expressed as AM ± SEM. The level of significance was set at *** (P<0.001), ** (P<0.01) and * (P<0.05).

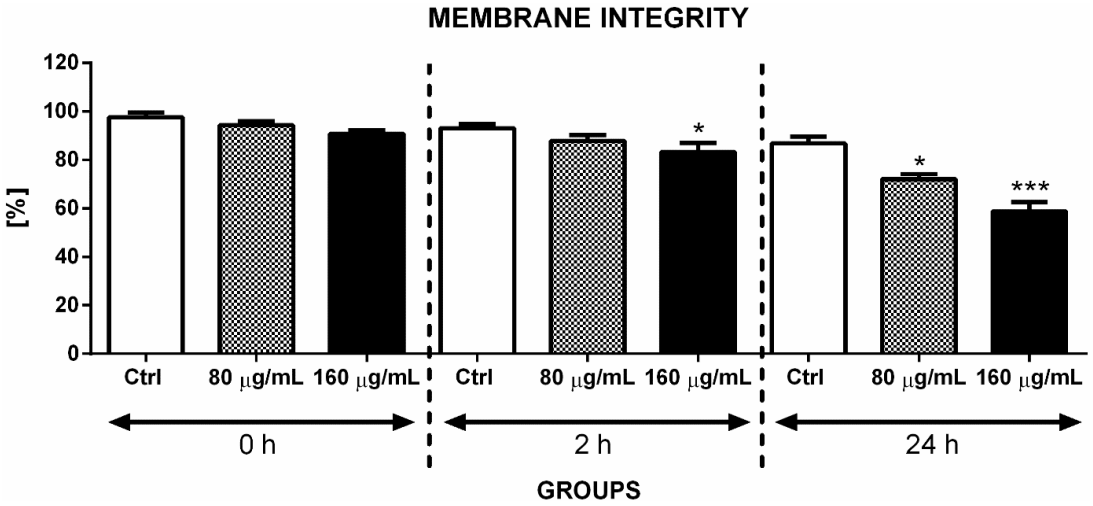

Figure 3. The development of the percentage of spermatozoa with intact membrane in the groups treated with 0 (Ctrl), 80 and 160 μg/mL during 24 h storage of *in vitro* culture. The results are expressed as AM ± SEM. The level of significance was set at *** (P<0.001), ** (P<0.01) and * (P<0.05).

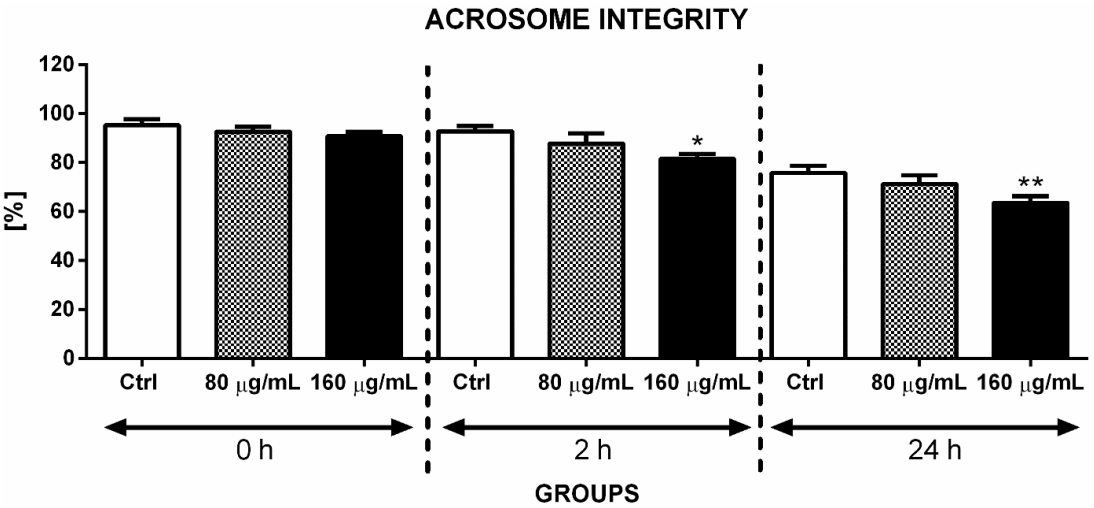

Figure 4. The development of the percentage of spermatozoa with intact acrosome in the groups treated with 0 (Ctrl), 80 and 160 μg/mL during 24 h storage of *in vitro* culture. The results are expressed as AM ± SEM. The level of significance was set at *** (P<0.001), ** (P<0.01) and * (P<0.05).

The results of mitochondrial activity assessment (Figure 2) showed the dose-dependent decreasing trend at the initial time with no significant differences when compared to Ctrl. The set decreasing trend continued also after 2 h with observable significant decrease in the group treated with 160 µg/mL of KAN. Finally, both concentrations of KAN significantly inhibited mitochondrial activity ($P<0.05$ in case of 80 µg/mL and $P<0.001$ in case of 160 µg/mL).

The eosin-nigrosin staining did not reveal any significant differences during the initial assessment (Figure 3). After 2 h, the percentage of sperm cells with damaged membrane integrity significantly increased in the group treated with 160 µg/mL. The results of final measurement showed significantly decreased percentage of sperm cells with intact membrane integrity in the group treated with 80 µg/mL ($P<0.05$) as well as the group treated with 160 µg/mL ($P<0.001$).

Similarly, the evaluation of acrosomal status (Figure 4) did not bring with any significant differences during the initial measurement. After 2 h, the percentage of sperm cells with damaged acrosomal integrity significantly increased in the group treated with 160 µg/mL. The dose-dependent trend was observed also after 24 hours. However, only in the group treated with 160 µg/mL significantly decreased ($P<0.01$) the amount of spermatozoa with intact acrosome.

4. Discussion

Lack of studies about the direct *in vitro* effect of KAN on spermatozoa gave rise to investigate basic structural and functional quality of bovine spermatozoa under these conditions. According to previous *in vivo* study on rats, aminoglycosides decreased the seminal vesicle weight. Moreover, production of abnormal spermatids was increased, depending on a dose, leading to increased sperm abnormalities. These structural abnormalities were reflected in a decreased sperm count and sperm motility. Decreased activities of superoxide dismutase, catalase and glutathione peroxidase in semen suggested that KAN may damage spermatogenesis and spermatozoa through inducing oxidative stress in the male reproductive tract **(Narayana, 2008)**. As unsaturated fatty acids are richly represented in mammalian sperm membranes, they are particularly sensitive to increased concentrations of reactive oxygen species (ROS), leading to the alteration of membrane fluidity **(Mahanta *et al.*, 2012)**, thus damage of membrane integrity.

Schlegel *et al.* (1991) reported that probably the premature termination of meiosis at the stage of primary spermatocytes may be responsible for harmful effects of aminoglycosides during spermatogenesis. On the other hand, the effect on mature spermatozoa was according to this study negligible. Furthermore, they reported that aminoglycosides had no direct effect on the viability and/or motility of spermatozoa under the *in vitro* conditions. Kanamycin, streptomycin, amikacin, gentamycin, tobramycin and neomycin had no detrimental effects at concentrations up to 1000 µg/mL. In contrast to this statement, our results proved detrimental effect of kanamycin at concentration of 160 µg/mL during the initial motility measurement. Other parameters, such as mitochondrial activity, membrane and acrosomal integrity, slightly decreased, but there was no significant difference.

Aminoglycosides can induce also programmed cell death. The formation of ROS begins with the entry of the aminoglycoside into the cell and binding with the iron cation to the aminoglycoside-Fe complex, which catalyzes the production of ROS in the presence of unsaturated fatty acids **(Lesniak *et al.*, 2005)**. One of the ROS-activated pathways is the c-Jun N-terminal kinase pathway, which translocates to the nucleus and activates genes responsible for apoptosis. Subsequently, a signal is sent to the mitochondria which induces the release of cytochrome c. Cytochrome c reports information to caspases on the initiation of cell death **(Davis, 2000; Rybak and Ramkumar, 2007)**.

Acrosomal integrity is largely influenced by the level of ROS. During physiological conditions, ROS play an important role in the fusion of male gamete with the oocyte **(El-Taieb *et al.*, 2015)**. However, if concentration of kanamycin is too high, then ROS are pathologically increased which leads to premature acrosomal reaction and therefore to the decrease of fertilizing potential of the semen sample.

Kalghatgi *et al.* (2013) studied detrimental side effects of bactericidal antibiotics in various cell lines. Clinically relevant doses of kanamycin had induced intracellular ROS production dependent from dose and time. Mitochondrial superoxide, a precursor of major forms of ROS, and concentration of released H_2O_2 were significantly increased in human MCF10A cell line. Therefore, we may hypothesize, that if there was the excess of kanamycin-induced ROS in sperm mitochondria, activity of succinate dehydrogenase was inhibited **(Andreyev *et al.*, 2015)**, thus the respiratory chain inhibited production of ATP resulting in decrease of spermatozoa motility.

4. Conclusion

Based on our findings, kanamycin had dose- and time-dependent effect on bovine spermatozoa under *in vitro* conditions. Concentration of 160 µg/mL of kanamycin had immediately detrimental effect on motility, which reflected after 2 h in decreased mitochondrial activity, damaged membrane and acrosomal integrity. There was only slight effect of kanamycin at concentration of 80 µg/mL immediately after mixing with spermatozoa. However, the use of kanamycin as a supplement during cryoconservation process could show in decreased total sperm quality during post-thaw process, and thereby could decrease fertilizing potential of bovine spermatozoa in the process of artificial insemination. Therefore, we do not suggest the use of kanamycin at observed concentrations as an antimicrobial agent in bovine semen preservation medium. The question remains whether lower concentrations of kanamycin would have the desired antimicrobial effect and at the same time would not damage the structural and functional properties of bovine spermatozoa.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Bee Pollen – Nutritional and Toxicological Aspects

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Abstract

Pollen contains nutritional elements – carbohydrates, proteins, lipids, vitamins, minerals, trace elements. The pollen contains also important amount of polyphenolic compounds, primarily antioxidants. Because the pollen is rich in nutrients, honeybee-collected pollen is recommended as a dietary supplement. The product can be used as dietary supplements to enrich food with valuable nutrients performing important functions in the human body. Pollen is a bee product affected by contaminants of various origins. In addition to important nutrients, it may also contain heavy metals that are harmful to human health. May be contaminated from air and soil by heavy metals and pesticides.

1. Introduction

The pollen gathered by honey bees (*Apis mellifera*) is considered like a potential source of energy for human nutrition. Pollen contains nutritional elements like carbohydrates, proteins, amino acids, lipids, vitamins, minerals and trace elements **(Serra Bonvehí and Escolá Jordá, 1997)**. Apart from this the pollen contains also important amount of polyphenolic compounds, primarily antioxidants **(Almeida-Muradian *et al.*, 2005).**
Bee-collected pollen is nutritionally valuable special food, having different health enhancing effects, and is also used in apitherapy **(Bogdanov, 2004)**. This bee product has several pharmaceutical effects, like antibiotic, antineoplastic, antidiarrheic and also as antioxidative agent **(Campos *et al.*, 1997)**.
This review aims to present significant knowledge about pollen and analyze selected nutritional and toxicological aspects of bee pollen.

2. Pollen, types of pollen

It is important to distinguish between three types of pollen:

a) natural, flower pollen, which releases from anthers and it is not process at all.
b) fermented, named also "bee bread", which was hydrolysed by non-reducing sugars and fermented by lactic acid in combs. This pollen is the most valuable in human nutrition.
c) corbicular pollen (bee pollen), which is collected by worker-bees from flowers and then is processed by bee's gland and honey sac secretions.

Pollen grains were characterized the first time in 1682 by Nohamiah Grew. The size of pollen grains is ranged from 2.5 to 250 μm, which have reproduction function and also other physiological functions. Bees form them into loads (size 1.5-2.5 mm) by glands secretions (bee pollen) and carry them on the corbiculas (pollen baskets) to a hive.
In human nutrition are used bee and fermented pollen. Fermented pollen is obtained from honey combs, but this is very laborious. For this reason is mostly used bee pollen. On other hand bee pollen missed some important elements e.g. enzymes, in comparison with fermented pollen, but its nutritional value is still very high. This deficiency can be complete by its consumption in honey **(Žitňanský, 1996)**.
Bee workers form pollen loads on the third pair of legs (corbiculas). They form them from thousand pollen grains which are put together with nectar and glands secretions. The bee pollen in comparison with flower pollen is sweeter and acquire other valuable features **(Chlebo and Čermáková, 2001)**.

Table 1. Elementary chemical composition of bee pollen (%) **(Schmidt and Buchmann, 1992)**

Compound	Average	Range
Proteins	23.7	7.5 - 35
Lipids	4.8	1 - 15
Sugars	27	15 - 45
Ash	3.12	1 - 5

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Table 2. Differences between bee and fermented pollen in % **(Dobrovoda, 1986)**

Type of pollen	Proteins	Lipids	Carbohydrates	Ash	Lactic acid	Active acidity
Bee pollen	24.06	3.33	18.5	2.55	0.56	6.3
Fermented pollen	20.3-21.7	0.67-1.58	24.4-34.8	2.4-2.6	3.06-3.20	4.3

3. Chemical composition and selected nutritional aspects

Chemical composition of bee pollen depends from plant species origin, from composition, moisture and fertility of soil, and also from climatic conditions during production and maturation of pollen grains in anthers. This explains differences in results pollen samples mentioned by different authors **(Dobrovoda, 1986)**. Contrary of other foods, bee pollen is not uniform product. Chemical composition of bee pollen varies widely. It differs on the cause of geographical origin, but also from one locality to other, or during the year. The bee pollen composition documented by tables 1 and 2.

Fresh, bee collected pollen contains about 20-30 g water per 100 g **(Bogdanov, 2004)**. The bee-collected product - pollen can be a good complement to daily diet due to the interesting proportions of proteins, fats and carbohydrates **(Orzáes Villanueva *et al.*, 2002)**.

Pollen (hand-collected or harvested by bees as pollen loads) has been a subject of study for many years because of its importance in bee nutrition and also because it provides a rich source of easily digestible protein and essential amino acids for humans **(Naumkin, 1984, Čeksterite, 1988, 1991; Campos *et al.*, 1996)**.

Protein content in pollen depends on the plant origin. Because of high protein content and rich amino acid composition, pollen has been the subject of numerous studies. The crude protein content has been determined to be close to 25 %. Many papers have reported these values that vary between a large range from 3.8 to 40.8% **(Naumkin, 1991; Serra Bonvehí *et al.*, 1991; Čeksterite, 1991; Serra Bonvehí and Escolá Jordá, 1997; Somerville, 1997)**. Pollen protein content has been the subject of studies conducted in Poland by **Syrocka and Zalewski (1985), Zalewski and Kosson (1985), and Szczęsna (2006a, 2006b, 2006c, 2007a, 2007b)**. Their results were higher than the findings of Spanish scientists (by Polish authors **Szczęsna *et al.*, 1995a** about 30%, according to Spanish studies by **Somerville, 1997** the crude protein level as low as 16 %) **(Serra Bonvehí *et al.*, 1986; Muniategui *et al.*, 1990; Serra Bonvehí *et al.*, 1991; Serra Bonvehí and Escolá Jordá, 1997)**. The crude protein content in the samples of bee-collected pollen by **Szczęsna (2006b)** ranged from 15.80 % DM (dry matter) for the pollen from Poland to 26.13 % DM for the pollen from China. Essential amino acids index EAAI reached value in the range from 97 % for pollen from Korea to 116 % for pollen from China (110 % on average). In all the pollen samples, methionine and cysteine have proven to be the limiting amino acids **(Szczęsna, 2006b)**, which was also found in earlier studies by other authors **(Naumkin 1991; Rogala and Szymaoe 2004)**. High value of essential amino acids index (EAAI=110 %) confirmed high nutritional value of pollen samples collected in Poland, Korea and China (**Szczęsna, 2006a)**. The similar results for CS and EAAI values was received by **Szczęsna (2006b)** for pollen from selected botanical origins. **Szczęsna (2006b)** investigated the protein and amino acid composition of honeybee-collected pollen from selected botanical origins. The crude protein content in the examined samples ranged from 13.06 % DM for *Artemisia* pollen to 24.54 % DM for that of *Sinapis alba*; the mean value was 20.55 % DM. The protein content found in the rapeseed pollen (*Brassica napus* subsp. *napus* L.) was on average 251.13 ± 33.06 $g.kg^{-1}$ **(Fatrcová-Šramková et al., 2008a, 2008b)**.

Carbohydrate fraction constitutes an essential part of honeybee-collected pollen dry matter. The first investigations on the subject were concerned with total sugar content, the distinction being made between reducing and non-reducing sugars **(Szczęsna *et al.*, 1995a)**. The study also showed that the basic sugar composition changes as the product is further processed, during its preservation (drying) and storage **(Szczęsna *et al.*, 1995b)**. **Szczęsna (2007b)** investigated the sugar composition in bee pollen collected in different countries (Poland, South Korea and China). The study showed that sugar content of pollen loads dry matter averages 40 %. As compared to pollen loads samples from South Korea and China those collected in Poland had a significantly higher fructose to glucose ratio. Of all the assayed sugars fructose was found to occur in the highest concentration. It accounted for 46 % of the total sugar content in the tested pollen samples. Glucose was the second highest accounting for 37 %. Monosaccharides expressed as the combined concentration of fructose and glucose accounted for 83 % of the carbohydrate fraction of pollen. When comparing the results from the study by **Szczęsna (2007b)** with those from earlier investigations **(Szczęsna *et al.*, 2002)** it can be seen that the data on the contents of individual sugars in the pollen samples differ considerably. The reason behind those differences is that the study by **Szczęsna (2007b)** was performed on pollen samples that came from different forage plants (different beekeeping seasons, different dates, different countries). The investigations of sugars in pollen loads showed that their minimum content was ca. 30 % DM, monosaccharides expressed as the sum of fructose and glucose accounting for at least 20 % DM.

It was estimated content of sugars in bee-collected pollen samples from *Papaver somniferum* L. Content of sugars was 340.7 $g.kg^{-1}$. Fructose content was 188.5 $g.kg^{-1}$, glucose content 152.2 $g.kg^{-1}$ and glucose/fructose ratio 0.81 **(Fatrcová-Šramková *et al.*, 2008c)**.

The content of the lipid fraction (the so-called “crude fat” or “ether extract”) determined by different authors was within a very wide range, depending on the species of plant the pollen originated from, i.e. from 1.5 % **(Standifer, 1966)** to 22.4 % **(Szczęsna *et al.*, 1995b)**. The content of the lipid fraction in the samples of honeybee-collected pollen originating from Poland ranged from 6.74 % DM to 10.99 % DM, i.e. 8.7 % DM on average, and was higher as compared with that reported for Korean (5.5 % DM) and Chinese (6.2 % DM) samples. The mean content of the lipid fraction in the samples from Korea was not statistically different from that of pollen samples from China. The study by **Szczęsna (2006c)** contributes to a precise determination of the composition and content of fatty acids of main pollen species obtained by beekeepers under Polish climatic and soil conditions.

According to different authors ash content of pollen varies from 1.55 % to 6.05 % **(Nation and Robinson, 1971, McLellan, 1977, Youssef *et al.*, 1978, Zalewski and Szymaniuk, 1985, Serra Bonvehí *et al.*, 1986, Szczęsna *et al.*, 1995a, Serra Bonvehí and Escolá Jordá, 1997)**. Polish authors **(Zalewski and Szymaniuk, 1985, Szczęsna *et al.*, 1995a)** found higher values for that constituent with an average of more than 3 % whereas Spanish authors reported values less than 2 % **(Serra Bonvehí and Escolá Jordá, 1997)**. Potassium, magnesium, calcium and sodium were found at the highest concentrations. Iron, manganese, zinc and copper were also relatively high. The investigators emphasize the dependence of the content of individual elements in pollen on its botanical origin.

Szczęsna (2007a) investigated the macro- and micro-elements composition (total ash and selected elements) of the multifloral honeybee-collected pollen harvested in different countries (Poland, South Korea and China). Ash content of the tested samples ranged from 2.08 to 3.19 % of DM for the samples from Poland, from 2.17 to 3.66 % DM for the samples from Korea and from 2.78 to 3.33 % DM for the samples from China. The following elements were assayed in the tested pollen samples: sodium, potassium, calcium, magnesium, manganese, zinc, iron and copper. Regardless of origin, potassium occurred at the highest concentrations in all tested pollen samples (59 % of the total content of minerals). The second largest with respect to content level was magnesium (18 %) followed by sodium (12 %) and calcium (8 %) and the remaining elements accounted jointly about 3 %. The contents of elements in the samples can be put in the following decreasing order: K > Mg > Na > Ca > Fe > Mn > Zn > Cu. It was found that contents of ash and of the minerals varied substantially from sample-to-sample which could be related to different botanical origin.

Melissopalynological analysis of the tested bee pollen samples allowed the placement of the samples within three groups: group I – samples with the predominance of pollen from Brassicaceae family (over 65 %), group II – samples with the predominance of pollen from the genus *Artemisia* (over 64 %), group III – samples of multifloral pollen originated from other ruderal plants (*Rumex*, *Coryphyllaceae*, *Ranunculus*, *Centaurea cyanus*, *Majoran*a type) and from agricultural and horticultural plants (*Rubus* type, *Fragaria*, *Trifolium* type), trees and shrubs (*Syringa*, *Cornus*, *Robinia*, *Salix*) **(Szczęsna, 2006b)**.

The pollen samples containing substantial percentage of pollen of *Artemisia* when compared to samples with a large percentage of *Brassicaceae* pollen and with samples of multifloral pollen were found to contain less ash and to be lower in all tested elements except calcium. The ash content of samples with substantial percentage of *Artemisia* pollen was 2.14 % DM whereas it was 2.71 % and 2.91 % in the two remaining groups, respectively. The ash content of 3% as determined in the study by **Szczęsna (2007a)** for multifloral pollen samples is in agreement with earlier studies both by the author **(Szczęsna *et al.*, 1995a)** and by other Polish investigators **(Zalewski and Szymaniuk, 1985)**. Spanish investigators obtained ash contents of pollen that were much lower being as low as ca. 2 % **(Serra Bonvehí *et al.*, 1986, Serra Bonvehí and Escolá Jordá, 1997)**.

The important part of bee pollen is water soluble vitamins from the group B: vitamin B1, vitamin B2, vitamin B6, pantothenic acid, folic acid, vitamin C. From vitamins soluble in fats are mostly represented by vitamin E and vitamin D **(Neuschlová, 1995)**.

4. Antioxidants, antioxidant and antiradical activity

Antioxidants, inclusive of flavonoids, beta-carotene, vitamin C and E, and selenium are also the part of bee pollen **(Schmidt and Buchmann, 1992)**. Antioxidants are considered as protective substances to free radicals which damage the human organism; and moderate a development of chronic diseases **(Gulcin *et al.*, 2003)**. Studies by **Campos (2000) and Campos *et al.* (2002)** are oriented to antioxidant properties of bee pollen, and they confirmed the high antiradical activity of bee pollen. It seems that antiradical activity is caused by phenolic components. In floral pollen mostly flavonoids, their glycosides and derivatives of cinnamic acid are present **(Markham and Campos, 1996)**. The antioxidant activity of bee pollen has been recognized as a free radical scavenger and as a lipid peroxidation inhibitor **(Campos *et al.*, 1997, Campos *et al.*, 1994)**.

The free radicals damage membranes of different organs cells, including the liver cells, what is considered as primary reason of organism aging. Rats, aged 23 months, were one time a day orally fed by bee pollen (250 $mg.kg^{-1}$) during one month. This treatment led to improving lipids peroxidation, antioxidant protective system and liver functions, which can relate to natural antioxidants (flavonoids) contained in bee pollen **(Uzbekova *et al.*, 2003)**. **Capcarová et al. (2013, 2019)** also dealt with bee pollen and consumption in rats in research.

Bee-collected pollen is an apicultural product which contains considerable amounts polyphenol substances ($8,2 \pm 0,3$ $mg.g^{-1}$) which may act as potent antioxidants. Bee-collected pollen extracts show considerable antiradical activity which is significantly increased in the pollen extracts. The highest degree of radical scavenging activity is found in the ethanol extract, which also has highest concentration of polyphenol substances. For this reason, it can be assumed that there is a general correlation between the content of total polyphenols and the antioxidant and radical scavenging activity of the pollen preparations. Thus, the prepared extracts of bee-collected flower pollen may be regarded as effective natural and functional dietary food supplement due to their remarkable content of polyphenol substances and significant radical scavenging capacity with special regard to their nutritional-physiological implications and their health promoting effect **(Kroyer and Hegedus, 2001)**. The antioxidants, antiradical, and antioxidant activity of bee pollen have been studied **(Fatrcová-Šramková *et al.*, 2008a-d, Kačániová *et al.*, 2010)**.

5. Selected toxicological aspects

Pollen is a bee product affected by contaminants of various origins. May be contaminated from air and soil by heavy metals and pesticides. For optimal pollen quality, it is recommended to collect pollen in areas at least 3 km away from the source of contamination, such as intensive traffic, industrial centers and pesticide-treated agricultural areas **(Bogdanov, 2004)**.

While being a natural source of bio-elements pollen can also contain elements harmful to human health: cadmium, lead, mercury and arsenic **(Free *et al.*, 1983, Lipińska and Zalewski, 1989, Migula, 1990, Konopacka *et al.*, 1993, Szczęsna *et al.*, 1993)**. Pollen loads are assayed for heavy metal contents mainly because of the contamination of environment with those metals. The authors of those studies report on ever more frequent cases of admissible contents of those health-compromising metals being exceeded and point to the need for their level in honeybee-collected pollen to be constantly monitored. Pollen production-oriented apiaries should not be located in heavily industrialized areas, in a close proximity of heavy traffic roads or close to large urban agglomerations.

Heavy metals concentrations were analysed in bee gathered (in case of three treatment: dried, frozen, freeze-dried bee pollen) and flower pollen samples. From the heavy metals, in flower and bee pollen, the lead level was 0.64 $mg.kg^{-1}$ and less than 0.1 $mg.kg^{-1}$, respectively. The contents of mercury were 0.019 $mg.kg^{-1}$ in the flower pollen and ranging from 0.004 to 0.005 $mg.kg^{-1}$ in the frozen, freeze-dried and dried bee pollen. The cadmium concentration in the flower pollen was 0.12 $mg.kg^{-1}$, and in the bee pollen ranged from 0.22 to 0.26 $mg.kg^{-1}$ **(Kačániová *et al.*, 2008a, b)**.

Various physical, chemical and biological methods can be used to determine environmental contamination, the most popular being the latest **(Madras-Majewska and Jasiński, 2005)**. Several researchers recommend using adult bee bodies and bee products to monitor environmental cleanliness **(Crane, 1984; Jędruszczuk, 1987; Roman, 1997, 1998, 2000; Muszyńska, 1995; Szczęsna *et al.*, 1993; Jabłoński *et al.*, 1995).** The honey bee is one of the major carriers of environmental information. In its way and character of life, it is very closely related to natural conditions and immediately responds to their change. For some properties (easy manipulation, relocation, precise detection

circuit, full-area monitoring in the field) it is a suitable object for testing **(Čermáková, 1997)**. In many countries, bees and bee products have been used to assess the degree of environmental pollution **(Loper *et al.*, 1980; Roman, 2000)**. Of bee products, bee pollen and bee bread are very often used as experimental material. Bee bread is a good indicator of contamination because it is exposed to direct contamination of the environment **(Madras-Majewska and Jasiński, 2005)**.

Bees, but especially the pollen and propolis, are a very good and sensitive bioindicator of the state of the environment. They can be used to monitor environmental pollution, especially toxic elements. They are used to monitor the occurrence of some chemical elements that pose a potential risk to humans. Such risk elements include aluminum, arsenic, antimony, beryllium, chromium, cadmium, nickel, lead, mercury, selenium. These elements may, at a certain concentration, endanger human health, and children are very sensitive. These elements inhibit the enzymatic activity in the body, do not disintegrate, do not undergo metabolic breakdown and accumulate in the body. They penetrate the body by inhalation, penetration (through the skin) and through the food chain **(Čermáková, 1997)**.

The contamination of bee products (nectar, pollen, honeydew, etc.) occurs through air, water, plants, soil, and also by transport to the beehive by bees. Bees and their products can contaminate air and soil containing heavy metals, mostly from industry and transport **(Bogdanov, 2005)**.

Monitoring and analysis of heavy metals in pollen have a long history. **Bogdanov (2005)** summarized a number of findings from published studies on pollen, beeswax and propolis contamination with heavy metals **(Altmann, 1983; Cesco *et al.*, 1994; MAFF, 1995; Leita *et al.*, 1996; Conti and Botre, 2001; Madras-Majewska and Jasiński, 2003)**. Values vary and range widely:

Lead: in honey 0,01 - 1,80 mg.kg^{-1}, in pollen 0,02 - 3,90 mg.kg^{-1}, in beeswax 0,06 - 6,20 mg.kg^{-1}, in propolis 0.003 - 461.0 mg.kg^{-1}.

Cadmium: in beeswax 0,01 - 0,10 mg.kg^{-1}, in honey 0,03 - 2,10 mg.kg^{-1}, in pollen 0.05 - 2.30 mg.kg^{-1}, in propolis 0.006 - 3.8 mg.kg^{-1}.

Bees can also serve with their products as bio-indicators of heavy metal contamination, especially lead **(Altmann, 1983; Cesco *et al.*, 1994)**. Lead and cadmium are considered to be the major toxic heavy metals and are thus the most studied subject **(Bogdanov, 2005)**.

The heavy metal content in the pollen was analyzed to assess the state of environmental contamination by these metals. **Kačániová *et al.* (2008a, 2010)** analyzed the presence of heavy metals (cadmium, mercury, lead) in bee pollen samples (after three treatments - after drying, freezing and lyophilizing pollen) and in flower pollen samples. The results of the experiments are presented in Table 3.

Table 3. Heavy metal content in flower and bee pollen (mg.kg^{-1}) **(Kačániová *et al.*, 2008a, 2010)**

Heavy metals	Flower pollen *	Bee pollen *	
Cadmium	0.120	dried	0.25
		frozen	0.22
		lyophilized	0.26
Mercury	0.019	dried	0.005
		frozen	0.004
		lyophilized	0.004
Lead	0.640	dried	< 0.1
		frozen	< 0.1
		lyophilized	< 0.1

* year 2007

Cadmium does not disintegrate in the soil, it remains in the soil, resulting in contamination of the plant mainly by simple diffusion through the root system. Cadmium is very mobile in plants. It binds to part of the protein molecule. Although pollen grains are male sex cells and are protected in flower buds, they may be contaminated. Research has shown that pollen is very suitable for the indication of cadmium because it contains many protein substances with cadmium-bound groups (with sulfhydryl groups SH-). There is currently a trend of increasing cadmium content in the environment. The sources of cadmium that have a negative impact on the human organism are, for example, waste water, industrial fumes, transport **(Šalgovičová and Zmetáková, 2006)**.

Kačániová *et al.* (2008a, 2010) report cadmium concentrations in flower pollen of 0.12 mg.kg^{-1} and bee pollen of 0.22 - 0.26 mg.kg^{-1} (Table 3). The cadmium content was lower in the flower pollen than in the bee pollen, but other heavy metals (lead and mercury) reached a higher content in the flower pollen. **Čermáková (1997)** found that in selected monitored areas (Bardejov, Spišská Nová Ves, Gelnica, Ružomberok and Bratislava) the maximum cadmium value in the pollen was measured in 1989 at 0.172 mg.kg^{-1}, while in 1995 the maximum value was 0.720 mg.kg $^{-1}$. Higher values were found in samples from agricultural production areas. **Patruica *et al.* (2008)** report the cadmium content of the fermented (mixed and sunflower) pollen presented in Table 4.

Mercury enters the atmosphere by evaporation from decaying minerals containing mercury, from volcanic gases and evaporation from the oceans. The most dangerous forms for the organism are mercury vapors and alkyl mercury compounds (methyl mercury) **(Čermáková, 1997)**. Exposure to mercury, which may be represented by an inorganic form and an organic form that is more toxic, may cause central nervous system disorders **(Šalgovičová and Zmetáková, 2006)**. High concentrations of mercury are harmful to humans or bees. Mercury contamination was found in pollen, nectar, honeydew, bee-collected products. The significant development of heavy industry and automobile transport has caused a high content of mercury in the environment **(Madras-Majewska and Jasiński, 2005)**. According to **Kačániová *et al.* (2008a, 2010)** the mercury content in the flower pollen was 0.019 mg.kg^{-1}. The range of 0.004 - 0.005 mg.kg^{-1} in bee pollen (Table 3) does not indicate differences between dried, frozen and lyophilized pollen. **Madras-Majewska and Jasiński (2005)** evaluated the mercury content of bee bread (in fermented pollen) from different regions of Poland. They state that if mercury contamination in selected areas is high, bee-bread contamination with this element is also high. Overall, it was observed that the mercury content in bee pollen was low and did not exceed the standards, while the reported limit for children and infants was according to the Ministry of Health in Poland **(Rozporządzenie Minister Zdrowia, 2003)** 0.01 mg.kg^{-1}. All samples of bee bread examined contained mercury. The content was 18.10^{-5} - 795.10^{-5} mg.kg^{-1}, the average content was 92.10^{-5} mg.kg^{-1}. A similar average mercury content in bee bread was observed by Żarski et al. (1996), namely 91.10^{-5} mg.kg^{-1}.

The concentration of lead in the environment is influenced by the amount of emissions from leaded gasoline engines, which represent 85 % of the total lead volume in the atmosphere. The decrease in lead content in the environment is not yet significant, although the positive impact of ecological petrol production is visible **(Čermáková, 1997)**. Adverse effects of lead on humans have been proven **(Šalgovičová and Zmetáková, 2006)**. According to **Kačániová *et al.* (2008a, 2010)** a lead content of 0.64 mg.kg^{-1} and bee pollen of less than 0.1 mg.kg^{-1} was found in the flower pollen (Table 3). **Patruica *et al.* (2008)** report the lead content of the fermented (mixed and sunflower) pollen, presented in Table 4.

Table 4. Heavy metal content (ppm) in different types of pollen **(Patruica *et al.*, 2008)**

Element	Mixed flower polen	Rapeseed pollen	Sunflower pollen	Mixed fermented pollen	Sunflower fermented pollen
Cadmium	0.011	0.015	0.006	0.006	0.002
Lead	0.010	0.008	0.006	0.003	0.003

Arsenic is found primarily in the form of silver, lead and copper sulfides. In the metallurgical industry, especially in the melting of copper, the concentrations of arsenic in the atmosphere and in the soil are significantly higher. It is similar in the combustion of younger coal. Toxicity of arsenic depends on its chemical form, mainly on arsenic ions (As^{3+}), which are more toxic than methylated. Prolonged exposure to arsenic can cause various diseases, for example, it can cause damage to the central nervous system. The arsenic content fluctuates during the season, it is higher in the spring months, then there is a decrease. This is probably due to the immission gradient after the winter heating season and the long-term transmission of immissions **(Čermáková, 1997)**.

Other authors also note that pollen is not only a natural source of minerals, but may also contain elements harmful to human health: cadmium, lead, mercury and arsenic **(Free *et al.*, 1983; Lipińska and Zalewski, 1989; Migula, 1990; Konopacka *et al.*, 1993; Szczęsna *et al.*, 1993)**. The authors draw attention to the frequent causes of exceeding the acceptable contents of these health-damaging elements and to the need for constant monitoring of their levels in the bee pollen. Pollen production hives should not be located in industrial areas, in close proximity to busy roads or large urban agglomerations **(Szczęsna, 2007a)**. The long half-life of metals, their accumulation in the body can cause long-term stress on the body and toxic effects. The toxicity of various metals can be varied by the interaction of metals and other substances. Selenium can reduce the toxicity of methylortute and zinc in turn the toxic effect of cadmium. At present, levels of toxic metal concentrations in the environment are relatively low, with the exception of areas with intensive industrial activity, so that direct damage to health may not occur during human life.

Systematic monitoring of the concentration of toxic metals in the atmosphere, soil, water and sanitary and technological-preventive measures is the primary method for reducing metals in the environment and thus in the food chain. The timely capture of increased amounts of contaminants in the environment is very important. Therefore, it is important to use pollen collected by bees as one of the sensitive bioindicators of environmental pollution **(Čermáková, 1997)**.

Table 5. Heavy metal content in pollen samples ($mg.kg^{-1}$) **(Chlebo and Čermáková, 2001)**

Heavy metals	Cadmium	Mercury	Lead	Arsenic	Chromium
1st analysis	0.180	0.018	1.700	0.605	0.650
2nd analysis	0.043	0.003	0.380	0.182	0.120
Limit	0.100	0.050	1.000	1.000	0.500

1st analysis: year 1990, 2nd analysis: year 1999

According to **Chlebo and Čermáková (2001)**, pollen contamination is threatened mainly by industrial pollutants and the application of plant protection products. In 1990 and 1999 the authors investigated the presence of selected chemical elements (Table 5) in sixteen pollen samples from four regions of Slovakia with high ecological load (Stredny Spis, Ruzomberok, Horna Nitra and Bratislava). In the given situation, the air quality in Slovakia was generally satisfactory. This was not only a reduction in production but also a greening of production for large exhaled producers. The risk of contamination by pesticide residues comes to the forefront when pollen sources are important crop plants such as rapeseed, sunflower and others.

6. Conclusion

In the world marked the bee pollen is mostly used as food supplement in the form of capsules, granules, tinctures, tonics, cereal bars, sweets etc. Partly it is used for animal feeding, above all bumblebees, honey bees and race-horses. Pollen has been shown to be an excellent dietary component in diets for specialty or valuable animals. Nowadays the possibilities of bee pollen utilization are underrated whereby manufacture potential exists. It would also be advisable to continue and extend investigations into the composition and contents of honeybee-collected pollen. Following studies are needed to enable the species-oriented production of bee pollen characterized by a high content of valuable nutrients.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Biogenic Amines in the Different Types of Cheese

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Abstract

The aim of the experiment was to analyse the content of biogenic amines (BA) in the main groups of commercially produced cheeses and in cheese matured under standard conditions. The content of biogenic amines in commercially produced cheeses was monitored on the last day of consumption, and on the 1st, 30th and 60th days after production in the cheese matured under standard conditions. The highest content of biogenic amines was found in semi- hard cheeses, mainly in Eidam slices (tyramine123.12 mg.kg^{-1} putrescine 23.02 mg.kg^{-1}). The cheese was not treated after the production and was cut and packed after production, which significantly affected the formation of biogenic amines. Also, "Salámový syr" - cheese had a high content of biogenic amines (tyramine 65.12 mg.kg^{-1} putrescine 29.25 mg.kg^{-1}). High content of BA was also found in the other semi- hard cheeses - "Hranol neúdený" (tyramine 56.02 and putrescine 51.49 mg kg^{-1}). In general, the content of biogenic amines in semi- hard cheeses is higher than in semi-soft, because they have a longer ripening time.

1. Introduction

Biogenic amines (BA) are low molecular weight aliphatic organic compounds derived from amino acids. They commonly participate in metabolic processes in living tissues **(Komprda, 2005).**
According to the chemical structure, BA are composed of aromatic (tyramine, phenylethylamine), heterocyclic (histamine, tryptamine), aliphatic (putrescin, cadaverine) and polyamines (spermidine, spermine, or agmatine) **(Velíšek, 2002; Čuboň *et al.,* 2017).**
Proteolytic processes, including the release of free amino acids from the protein matrix, result mainly from the activity of the enzymatic apparatus of the SLAB and NSLAB present. The product of histidine decarboxylation is histamine, and cadaverine is formed from lysine **(Buňka *et al.,* 2012; Pachlová *et al.,* 2015).**
The BA content in certain foods may be very different. Typical levels of biogenic amines in foods range from 10 mg.kg^{-1} to 100 mg.kg^{-1}. BA content detected in food can exceed 1000 mg. kg^{-1} **(Buňková, 2010).** The BA concentration in fresh cow milk is less than 1 mg.kg^{-1}. These are primarily histamine and tyramine. The histamine content in milk varies from 0.5 to 0.8 mg. kg^{-1}, the histamine content of the dried milk is around 131 mg. kg^{-1}, tyramine content 42 mg.kg^{-1}. The BA content in the cheese may be higher than10 g.kg^{-1} **(Greif and Greifová, 2006).**
BA content of all cheeses gradually increases during ripening process. Their kinetics also depends on the type of cheese and the technology used. Hard cheeses contain less BA than soft cheeses. The highest values were in the tyramine content (up to 146 mg.kg^{-1}) and histamine up to 85 mg.kg^{-1}. Also, tryptamine, phenylethylamine, putrescine, cadaverine, spermine, spermidine, adrenaline and noradrenaline were identified in the cheeses **(Kolesarová, 1995).**
The aim of the experiment was to analyse the content of biogenic amines in the main groups of commercially produced cheeses and in cheese matured under standard conditions.

2. Material and methods

In our study, a total of 40 samples of natural cheeses were analysed. They were purchased from various stores in the Slovak Republic.
The cheeses were divided according to the consistency (expressed as the water content in the non-fatty matter of the cheese - VBHS%) into groups (hard 49-56%, semi- hard 54-63%, semi-soft 61-69%, and soft 67%).
In the experiment, the following samples of cheese were analyzed according to the following consistency:
- Hard cheese: A - Primator (n = 5),
- Semi- hard cheese: B - Eidam slices (n = 5), C- „Salámový syr neúdený"- cheese (n = 5), D- „Hranol neúdený"–cheese (n = 5),
- Semi - soft cheeses: E - Mold cheese (n = 5), F - Half-horsepowder (n=5),
- Soft cheese: G- Plesnivec (n = 5), H-Encian (n = 5).
The cheese was analysed at the end of expiration date. From the purchase until the analysis, the samples were stored at 10 ± 2 °C.

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2.1 Ripening experiment

In the second part of the experiment, an Eidam brick was produced in a dairy plant (n = 5) from five milk samples. The cheese was produced to monitor the content of biogenic amines during maturation. Samples for analysis were taken on 1st, 30th and 60th day after production. They were stored at 10 ± 2 °C all the time.

In the study, there are limits for analysed parameters (mg.kg^{-1}) for a complex matrix, such as: histamine - 1.07, tyramine - 1.41, putrescin - 1.65, cadaverin - 1.72, agmatin - 2.54, spermidine - 0.71, spermin - 1.57. The actual detection limits for biogenic amine standards are approximately at a quarter of these values.

Free amino acids and biogenic amines were determined by using ion-exchange chromatography (AAA400 Amino Acid Analyzer; Ingos, Prague, Czech Republic) according to **Buňková *et al.* (2010).** Each cheese sample was analysed twice. Standards were supplied by Sigma–Aldrich.

The data were processed in statistical analysis using the Statistic Analysis System (SAS) package (SAS 9.3 using of application Enterprise Guide 4.2).

3. Results and discussion

In commercial cheese production in the Slovak Republic, concentrations of biogenic amines (histamine, tyramine, putrescin, cadaverine, agmatine, spermidine, spermine) were analysed by an ion exchange chromatography method using the AAA400 analyser. The cheeses were analysed at the end of the expiration date.

The content of biogenic amines in extruded cheeses is shown in Table 1.

In the hard cheese (Primator) the tyramine content was 11.05 mg.kg^{-1} and putrescine 11.15 mg.kg^{-1}. Other analysed biogenic amines were below the limit of analysis (Table 1). In semi- hard cheese – „Eidam slices", tyramine content was 123.12 mg.kg^{-1} and putrescine 23.02 mg.kg^{-1}. In „Salámový syr neúdený" - cheese, tyramine content was 65.12 mg.kg^{-1}, putrescine 11.05 mg.kg^{-1} and cadaverine 11.05 mg.kg^{-1}. In the „Hranol neúdený" –cheese tyramine content was 56.02 mg.kg^{-1} and putrescine 51.49 mg.kg^{-1}.

In semi soft cheese – „Plesňový syr" cheese, the tyramine content was 24.05 mg.kg^{-1} and putrescine 21.62 mg.kg^{-1}. In the „Gazdovský polooštiepok" - cheeses, only the cadaverine content of 3.25 mg.kg^{-1} was found. Other biogenic amines levels were under the analysis limit.

Higher contents of tyramine - 180.4 mg.kg^{-1} in the cheese is reported by **Buňková (2010).** However, tyramine content in the cheese can also reach 500 mg.kg^{-1} **(Leuschner *et al.,* 1999).** The highest content of biogenic amines was found in semi- hard cheeses mainly in Eidam slices (tyramine - 123.12 mg.kg^{-1}, putrescine - 23.02 mg.kg^{-1}). The cheese was not heat-treated after production and was cut and packaged after production, which significantly affected the formation of biogenic amines. Also, „Salámový syr neúdený"- cheese had a high content of biogenic amines (tyramine - 65.12 mg.kg^{-1}, putrescine - 29.25 mg.kg^{-1}). The high content of BA was also in the other „Hranol neúdený"- cheese (tyramine - 56.02 and putrescine - 51.49 mg.kg^{-1}). Generally, the content of biogenic amines in semi- hard cheeses is higher than that of semi-soft cheese because they have a longer maturing time.

Soft cheeses including „Bryndza"– cheese had higher concentration of biogenic amines (300 mg.kg^{-1}) in spring bryndza **Greif, Greifová (2006)**.

Also, **Dičaková and Dudriková (2006)** found high tyramine content (52.4-410 mg.kg^{-1}) and also indicate that the content of biogenic amines in food is also increasing during storage of products.

In comparison to our results, **Standarova *et al.* (2008)** found significantly higher concentrations of biogenic amines in soft ripened cheeses. The highest concentrations were also found in the „Bryndza"- cheese (417 mg.kg^{-1} of tyramine, 591 mg.kg^{-1} of putrescine and 1110 mg.kg^{-1} of cadaverine) and „Hermelín" - cheese (187 mg.kg^{-1} of tyramine).

In the second part of the experiment, an Eidam brick was produced in a dairy plant (n = 5). The content of biogenic amines was analysed during maturation (on the 1st, 30th and 60th day after production). Cheeses were stored at 10 ± 2 ° C throughout the maturing time. The observed concentrations of biogenic amines are shown in Table 2.

During the 60 days of Eidam brick cheese maturation at 10 ± 2 °C, the content of biogenic amines was analysed. No biogenic amines were found in samples on the 1st day post-production. On the 30th day after processing, we found tyramine content of 8.256 mg.kg^{-1}, putrescine of 4.81 mg.kg^{-1} and cadaverine of 12.22 mg.kg^{-1}. The content of histamine, agmatine, spermidine and spermine was below the detection limit.

In cheeses on the 60th day after processing, we found tyramine content of 14.25 mg.kg^{-1}, putrescine of 10.25 mg. kg^{-1}, cadaverine of 26.42 mg. kg^{-1} and spermidine of 1.31mg. kg^{-1}. Other analysed biogenic amines (histamine, agmatine and spermine) were below the detection limit.

Pachlová *et al.* (2018) did not detect the content of biogenic amines in cheeses with microorganism without decarboxylation activity. But when using *Lb. paracease DEPE T52,* content of biogenic amines on the 30th day was 61.4 mg. kg^{-1} and on the 60th day 77.4 mg. kg^{-1}. Tyramine content on the 30th day was 20.6 mg.kg^{-1} and 29.9 mg.kg^{-1} on the 60th day.

Table 1. Content of biogenic amines in commercialized cheeses (mg.kg^{-1})

Cheese	HIS	TYR	PUT	CAD	AGM	SPD	SPM
Hard cheese **A**	ND	11.05 ± 1.09	11.15 ± 0.95	ND	ND	ND	ND
Semi- hard cheese **B**	ND	123.12 ± 9.02	23.02 ± 0.94	ND	ND	ND	ND
C	ND	65.12 ± 4.21	29.25± 1.42	11.05±0.45	ND	ND	ND
D	ND	56.02 ± 1.72	51.49 ± 2.82	ND	ND	ND	ND
Semi - soft cheese **E**	ND	24.05 ± 2.48	21.62 ± 0.98	ND	ND	ND	ND
F	ND	ND	ND	3.25 ± 0.45	ND	ND	ND
Soft cheese **G, H**	ND	ND	ND	ND	ND	ND	ND

Legend: HIS – Histamine, TYR - Tyramine, PUT - Putrescine, CAD – Cadaverine, AGM – Agmatine, SPD – Spermidine, SPM – Spermine, ND = non detected

Table 2. The biogenic amines contents in the Eidam brick in the course of maturing (mg.kg^{-1})

HIS	TYR	PUT	CAD	AGM	SPD	SPM
1^{-st} day						
ND	ND	ND	ND	ND	ND	ND
30^{-th} day						
ND	8.25 ± 0.62	4.81 ± 0.48	12.22 ± 0.51	ND	ND	ND
60^{-th} day						
ND	14.25 ± 0.62	10.25 ± 0.91	26.42 ± 0.95	ND	1.31 ± 0.21	ND

Legend: HIS – Histamine, TYR - Tyramine, PUT - Putrescine, CAD – Cadaverine, AGM – Agmatine, SPD – Spermidine, SPM – Spermine, ND = non detected

Conclusion

The formation of biogenic amines is affected by several factors. The microflora and production technology used have a great impact. The highest content of biogenic amines was found in semi- hard cheeses, mainly in Eidam slices (tyramine - 123.12 mg.kg^{-1}, putrescine - 23.02 mg.kg^{-1}). The cheese was not heat-treated after production and was cut and packaged after production, which significantly affected the formation of biogenic amines. Also, „Salámový syr neúdený"- cheese and „Hranol neúdený" had a high content of biogenic amines. In general, the content of biogenic amines in semi- hard cheeses is higher than in semi-soft, because they have a longer maturing time. At the experiment during the maturation we found approximately two times the increase of tyramine, putrescine and cadaverine from the 30th to the 60th day of maturing.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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