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ORAL AND INTRAMUSCULAR APPLICATION OF CYANOGENIC GLYCOSIDE AMYGDALIN DID NOT INDUCE CHANGES IN HAEMATOLOGICAL PROFILE OF MALE RABBITS

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ABSTRACT

Amygdalin is a cyanogenic glycoside initially obtained from the seeds of bitter almonds. It is composed of one molecule of benzaldehyde, two molecules of glucose and one molecule of hydrocyanic acid. Various ways of amygdalin application play a different role in recipient organism. Intravenous infusion of amygdalin produced neither cyanidemia nor signs of toxicity, but oral administration resulted in significant blood cyanide levels. The present *in vivo* study was designed to reveal whether amygdalin is able to cause changes in the haematological profile and thus alter the physiological functions, using rabbits as a biological model. Adult male rabbits (n = 20) were randomly divided into five groups: the control group without any amygdalin administration, two experimental groups received a daily intramuscular injection of amygdalin at a dose 0.6 and 3.0 $mg.kg^{-1}$ b.w. and other two groups were fed by crushed apricot seeds at dose 60 and 300 mg. kg^{-1} b.w., mixed with commercial feed over the period of 14 days. After two weeks, haematological parameters in whole blood were analysed (WBC – total white blood cell count, LYM - lymphocytes count, MID – medium size cell count, GRA – granulocytes count, RBC – red blood cell count, HGB – haemoglobin, HCT – haematocrit, MCV – mean corpuscular volume, MCH – mean corpuscular hemoglobin, MCHC – mean corpuscular hemoglobin concentration, RDWc – red cell distribution width, PLT – platelet count, PCT – platelet percentage, MPV – mean platelet volume, PDWc – platelet distribution width) using haematology analyser Abacus junior VET. Our findings indicate that intramuscular and oral application of amygdalin for two weeks did not significantly affect the haematology parameters in experimental animals. In this study, no obvious beneficial or negative effects of amygdalin administration on the blood of male rabbits were observed.

Keywords: apricot seed; amygdalin; haematology; rabbit

INTRODUCTION

Since ancient times, plants have been exemplary source of medicine **(Grover et al., 2002)** and have played key roles in traditional health care systems and also form the basis of a significant percentage of allopathic and modern drugs in industrialised nations of the world **(Calson, 1998; Samy and Gopalakrishnakone, 2007)**. Fruit and vegetable contain a significant amount of biologically active substances able to lower a risk of any type of cancer or other civilization diseases **(Mendelová et al., 2016; Jakubcova et al., 2014)**. Cyanogenic glycosides are plant secondary metabolites which consist of an aglycone and a sugar moiety. They are widely distributed in the plant kingdom, being present in more than 2500 plant species. Cyanogenic glycoside amygdalin can be found in plant families of the *Caprifoliaceae, Mimosaceaw, Oleaceae* and *Rosaceae*. It is abundant in the seeds of bitter almond and apricots of the *Prunus* genus **(Vetter, 2000; Fukuda et al., 2003)**. For more than 40 years, amygdalin has been one of the most popular "alternative cancer cures" in many European and South American countries **(Hwang et al., 2008)**. It has been isolated in 1830 by the French chemists Robiquet and Boutron-Charlard from kernels of the bitter almond (*Prunus amygdalus*) and has been thoroughly investigated in 1837 by Liebig and Wöhler. Its detailed chemical structure was at last established by the carbohydrate chemists Haworth and Wylam in 1923 **(Rauws et al., 1982)**. Amygdalin, is composed of two molecules of glucose, one of benzaldehyde, which induces an analgesic action, and one of hydrocyanic acid, which is an anti-neoplastic compound **(Chang et al., 2006)**. Amygdalin is sometimes confused with laevomandelonitrile, which is commonly known as laetrile. However, amygdalin and laetrile are different chemical

compounds **(Andrew et al., 1980; Du et al., 2005)**. Many studies have reported that amygdalin can be effectively used for prevention and treatment of various diseases including cancers, migraine, chronic inflammation, relieve fever and pain **(Fukuda et al., 2003; Yan et al., 2006; Zhou et al., 2012)**. Still, evidence based research on amygdalin is sparse and its benefit controversial. Proponents consider amygdalin a natural cancer cure, whereas opponents warn that amygdalin is ineffective and even toxic. Although it has been argued that amygdalin is unsafe, no serious acute toxicity has been encountered **(Milazzo et al., 2007)**. Amygdalin itself is non-toxic, but its production of hydrocyanic acid decomposed by some enzymes is poisonous substance **(Suchard et al., 1998)**. *In vivo* the enzyme complex emulsin containing the enzymes β-D-glucosidase, benzocyanase, and others, degrades the amygdalin into four components: hydrocyanic acid, benzaldehyde, prunasin, and mandelonitrile, which are absorbed into the lymph and portal circulations **(Chang and Zhang, 2012)**. Various ways of amygdalin application play a different role in recipient organism, what was confirmed by **Moertel et al. (1981)**, who demonstrated in human, that intravenous infusion of amygdalin produced neither cyanidemia nor signs of toxicity, but oral administration resulted in significant blood cyanide levels. The present *in vivo* study was designed to reveal whether amygdalin is able to cause changes in the haematological profile and thus alter the physiological functions, using rabbits as a biological model.

MATERIAL AND METHODOLOGY

Chemicals

Amygdalin from apricot kernels (≥99% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Amygdalin was freshly dissolved in sterile saline and 0.5 ml were applied intramuscularly (IM) to musculus biceps femoris on a daily basis. Bitter apricot seeds were provided by Trasco (Žiar n. Hronom, Slovakia). Thin Layer Chromatography (TLC) was performed for the analysis of amygdalin content in bitter apricot seeds used in our experiment. Grinded apricot seeds (2 g) were mixed with 10 mL of methanol in a vial and put into ultrasonic bath for 30 minutes at 55°C. After cooling, 10 μL of solution was applied onto TLC plates Kieselgel UV 254 20x20 cm (Merck KGaA, Darmstadt, Germany). Mixture of n-butanol, acetic acid and water (95 : 5 : 25) was used as a mobile phase. Separation took about 5 hours at room temperature. After separation, amygdalin content was determined by UV densitometer CS – 9000 (Shimadzu, Japan) at 205 nm. An external standard was used (1% amygdalin solution in methanol). Crude protein content was performed according to **Kjeldahl (1883)**, fat content was determined using Soxhlet method for fat extraction (1879) and crude fibre by Henneberg-Stohmann method. Amount of starch was measured via polarimetry and total sugars by Luff-Schoorl titration. Organic composition of the apricot seeds is shown in Table 1.

Animals

Male rabbits (n = 20), meat line P91 Californian from the experimental farm of the Animal Production Research Centre Nitra (Slovak Republic) were used in the experiment. The rabbits were 150 days old, weighing 4.00 ±0.5 kg, and were housed in individual flat-deck wire cages under a constant photoperiod of 12 h of daylight, temperature 20 – 24 °C and humidity 55% ±10%. The rabbits were fed a standard commercially available feed (Table 2) based on a pelleted concentrate. Animals had free access to feed and water during the study period and no toxic or side effects or death were observed throughout the study. The animals were randomly divided into the five groups, leading to 4 male rabbits in each group. The control group received no amygdalin while the two experimental groups E1 and E2 received a daily intramuscular injection of amygdalin at a dose 0.6 and 3.0 $mg.kg^{-1}$ b.w. respectively during 14 days. The experimental groups E3 and E4 were fed by crushed apricot seeds, at dose 60 and 300 $mg.kg^{-1}$ b.w., mixed with commercial feed for rabbits during the same period. Institutional and national guidelines for the care and use of animals were followed appropriately, and all experimental procedures were approved by the State Veterinary and

Table 1 Organic content in apricot seeds (%).

Dry mater	Amygdalin	Crude protein	Fat	Fiber	Starch	Sugar
95.9	5.2	22.8	39.7	28.5	2.3	6.3

Table 2 Chemical composition ($g.kg^{-1}$) of the experimental diet.

Component	
Dry matter	926.26
Crude protein	192.06
Fat	36.08
Fibre	135.79
Non-nitrogen compounds	483.56
Ash	78.78
Organic matter	847.49
Calcium	9.73
Phosphorus	6.84
Magnesium	2.77
Sodium	1.81
Potassium	10.94
Metabolizable energy	12.35 $MJ.kg^{-1}$

Food Institute of Slovak Republic, no. 3398/11-221/3 and Ethic Committee.

Blood sample collection and haematology analysis

Blood samples from *vena auricularis* were taken to tubes treatment with EDTA from all animals by macromethods after two weeks of experiment. In whole blood, haematological parameters were analysed (WBC – total white blood cell count, LYM – lymphocytes count, MID – medium size cell count, GRA – granulocytes count, RBC – red blood cell count, HGB – haemoglobin, HCT – haematocrit, MCV – mean corpuscular volume, MCH – mean corpuscular hemoglobin, MCHC – mean corpuscular hemoglobin concentration, RDWc – red cell distribution width, PLT – platelet count, PCT – platelet percentage, MPV – mean platelet volume, PDWc – platelet distribution width) using haematology analyser Abacus junior VET (Diatron MI LtD., Budapest, Hungary). The impedance method counts and sizes cells by detecting and measuring changes in electrical impedance when a particle in a conductive liquid passes through a small aperture. Each cell passing through the aperture – where a constant DC current flows between the external and internal electrodes – causes some change in the impedance of the conductive blood cell suspension. These changes are recorded as increases in the voltage between the electrodes. The number of pulses is proportional to the number of particles. The intensity of each pulse is proportional to the volume of that particle.

Statistical analysis

The data used for statistical analysis represent means of values obtained in blood collection. To compare the results, one-way ANOVA test was applied to calculate basic statistic characteristics and to determine significant differences among the experimental and control groups. Statistical software SIGMA PLOT 12.0 (Jandel, Corte Madera, CA, USA) was used. Differences was compared for statistical significance at the levels p <0.001, 0.01 and 0.05.

RESULTS AND DISCUSSION

Natural plant origin products like amygdalin are still a major part of traditional medicine. However, its effect on animal and human organisms is still not clear. **(Nabavizadeh et al., 2011; Kovacova et al., 2016)**. It has been used as a traditional drug because of its wide range of medicinal benefits, including curing or preventing cancer, relieving fever, suppressing cough, and quenching thirst **(Zhou et al., 2012)**. In addition, it can be used as a cerebral function improver that is effective as a therapeutic agent for cerebrovascular lesions such as psychogenic symptoms, nerve symptoms, subjective symptoms, and daily life activity disorder **(Hiromi, 1995)**. Previous studies on amygdalin have focused on its purification, toxicity related to the release of cyanide, anti-tumor mechanism, and identification of its metabolites in plasma or herbs, and its pharmacological effect on cancers **(Rauws et al., 1982; Yildirim and Askin, 2010; Makarević et al., 2014)**. It has been known that the harmful effects of amygdalin are directly related to the toxicity of cyanide, which is released following hydrolysis of the parent compound **(Newton et al., 1981)**. Haematological studies have been found useful for disease prognosis and for the therapeutic and feed stress monitoring **(Togun and Oseni, 2005)**. Haematological studies are important because the blood is the major transport system of the body, and evaluations of the haematological profile usually furnishes vital information on the body's response to injury of all forms, including toxic injury **(Schalm et al., 1975; Coles, 1986; Ihedioha et al., 2004)**. Haematological studies represent an useful process in the diagnosis of many diseases as well as investigation of the extent of damage to the blood **(Onyeyili et al., 1991)**. There are just a few studies of blood chemistry changes and changes in haematology profile after amygdalin administration *in vivo*. Our previous study did not confirm a negative effect on the energetic and hepatic profile of rabbits *in vivo* **(Tušimová et al., 2016a, Tušimová et al., 2016b)**. At the beginning of the experiment, before treatment with amygdalin and apricot seeds, all haematological parameters were in physiological range. On base of these results we could state that all animals were in good health condition. Haematological parameters of rabbits after two weeks of oral and intramuscular application of amygdalin are shown in Table 3.

The highest count of WBC was observed in E4 group where rabbits were fed by crushed apricot seeds, at dose 300 $mg.kg^{-1}$ b.w., mixed with commercial feed (equivalent to 15.6 $mg.kg^{-1}$ of amygdalin, equivalent to 0.92 $mg.kg^{-1}$ of HCN – hydrogen cyanide). In this group we also observed the highest count of MID among the groups. In case of GRA we found lower count of this kind of cells in all experimental groups in comparison with the control group. However the results were not significant and they were in physiological reference range for WBC, MID and GRA in rabbit blood. The total WBC can be used to further characterize acute stress from chronic stress (e.g., malnutrition, inproper husbandry, prolonged social stress, dental disease), as both a leukopenia and lymphopenia are more common with chronic stress **(Melillo, 2007)**. Higher WBC count may explain the reason for disease resistance which has been reported by **Nwosu (1979)** or the prevalence of disease condition. It may also explain longevity as reported by **Mbanasor et al. (2003)**. Lower count of GRA could by caused in acute inflammation, acute infection and intoxication **(Vrzgula et al., 1990)**. **Olafadehan et al. (2010)** carried out a study on the effect of residual cyanide in processed cassava peel meal on haematological indices of growing rabbits and observed that with exception of neutrophil and eosinophil, other haematological parameters were significantly affected by the dietary treatments. Amygdalin can significantly increase polyhydroxyalkanoates (PHA) induced human peripheral blood T lymphocyte proliferation and can promote peripheral blood lymphocytes stimulated by PHA secrete IL-2 and IFN-g, and then inhibit the secretion of TGF-β1, therefore enhance immune function **(Baroni et al, 2005)**. Amygdalin plays a positive role in the expression of regulatory T-cells in the treatment of atherosclerosis, and can also expand the lumen area, reduce aortic plaque coverage **(Jiagang et al., 2011**; **Perez, 2013)**. In our *in vivo* study, the highest LYM count in rabbit blood after addition of amygdalin was observed

Table 3 Haematological parameters of rabbits after oral and intramuscular application of amygdalin.

Parameter	C	E1	E2	E3	E4
WBC ($10^9.L^{-1}$)	10.78 ±3.21	11.12 ±0.97	11.01 ±1.85	10.67 ±2.54	12.29 ±3.92
LYM ($10^9.L^{-1}$)	2.91 ±0.90	3.16 ±0.75	4.31 ±0.71	3.36 ±0.89	2.85 ±1.20
MID ($10^9.L^{-1}$)	0.36 ±0.21	0.70 ±0.41	0.45 ±0.28	0.62 ±0.37	1.15 ±0.44
GRA ($10^9.L^{-1}$)	7.51 ±2.87	6.83 ±2.59	5.60 ±2.44	6.70 ±2.43	6.78 ±0.83
RBC ($10^{12}.L^{-1}$)	7.11 ±0.22	7.00 ±0.28	6.40 ±0.25	6.89 ±0.36	6.72 ±0.38
HGB ($g.L^{-1}$)	135.8 ±11.79	137.3 ±8.66	130.3 ±4.43	132.3 ±3.60	132.3 ±4.79
HCT (%)	43.97 ±3.03	43.10 ±2.56	41.53 ±1.88	42.26 ±0.68	42.40 ±1.42
MCV (fl)	61.75 ±2.63	61.50 ±2.08	64.75 ±2.06	61.25 ±2.75	63.00 ±2.16
MCH (pg)	19.05 ±1.17	19.63 ±0.77	20.33 ±0.19	19.30 ±0.80	19.70 ±0.96
MCHC ($g.L^{-1}$)	308.2 ±6.60	318.5 ±5.45	313.5 ±11.48	314.0 ±10.23	311.5 ±3.70
RDWc (%)	16.28 ±0.92	16.08 ±0.33	15.88 ±0.71	16.73 ±1.41	16.25 ±0.81
PLT ($10^9.L^{-1}$)	313.8 ±106.8	285.0 ±149.5	326.0 ±106.9	171.0 ±59.8	252.75 ±108.4
PCT	0.23 ±0.07	0.21 ±0.09	0.23 ±0.05	0.13 ±0.04	0.19 ±0.07
MPV (fl)	7.30 ±0.22	7.40 ±0.56	7.23 ±0.74	7.30 ±0.44	7.38 ±0.67
PDWc (%)	35.35 ±0.58	34.50 ±1.66	34.80 ±1.61	35.03 ±1.20	35.28 ±2.10

Note: WBC, total white blood cell count; LYM, lymphocytes count; MID, medium-size cell count; GRA, granulocytes count; RBC, red blood cell count; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDWc, red cell distribution widt; PLT, platelet count; PCT, platelet percentage; MPV, mean platelet volume; PDWc, platelet distribution width. C – control group, without addition of amygdalin; E1 and E2 experimental groups - intramuscular injection of amygdalin at a dose 0.6 and 3.0 $mg.kg^{-1}$ b.w.; E3 and E4 experimental groups - crushed apricot seeds, at dose 60 and 300 $mg.kg^{-1}$ b.w. The values shown are the mean ±SD (standard deviation).

in E2 experimental group when compared with the control and with the other experimental groups but the results were not significant. In E2 experimental group animal received amygdalin intramuscularly in concentration 3 $mg.kg^{-1}$ b.w. (equivalent to 0.177 $mg.kg^{-1}$ of HCN). Higher count of LYM may by due to intoxication and chronic infectious diseases **(Vrzgula et al., 1990)**. RBC count, content of HGB and HCT in rabbit blood were not singnificantly affected by treatment with amygdalin however the values of these parameters were the lowest in E2 experimental groups, when compared with the control and the other experimental groups. This values were still in physiological reference range for RBC, HGB and HCT in rabbit blood. In another fourteen-day toxicity study in rats HGB significantly increased after treatment with 20 $mg.kg^{-1}$ of amygdalin **(Oyewole and Olayinka, 2009)**. Lower values of RBC, HGB and HCT may indicate anemia **(Melillo, 2007)**. The lowest count of PLT and the lowest value of PCT were observed in E3 experimental group among the groups. In this group animals received apricot seeds in concentration 60 $mg.kg^{-1}$ b.w. in rabbit feed (equivalent to 3.12 $mg.kg^{-1}$ of amygdalin, equivalent to 0.184 $mg.kg^{-1}$ of HCN). These results were not significant and the values of this parameters were in physiological reference range. **Liu et al. (2012)** observed that Taoren–Honghua herb (TH) and its main components amygdalin and HSYA (hydroxysafflor yellow A) could significantly reduce platelet aggregation and protect vascular endothelial cells. Based on these results, amygdalin and HSYA were responsible for the main curative effects of TH and usually had synergetic effects, such as decreasing platelet aggregation percentage. Platelet aggregation is thought to be one of the factors that determine blood viscosity **(Ryu et al., 2009)**. Lower count of PLT may be due to intoxication **(Vrzgula et al., 1990)**. We did not observe any changes in the other analysed haematological parameters. In two feeding experiments (respectively 63 and 56 days) one day old broiler chickens (male and female) were fed a diet containing 0, 10, 20 or 30% cassava, respectively. Cassava also contains cyanogenic glycosides. The animals were studied for haematological and histopathological effects. No changes in the haematological parameters due to cassava were seen **(Gomez et al., 1988)**.

No clinical signs were observed in short-term studies and changes in haematology were minor, sporadic and not dose related. In none of the reported short-term studies was mortality observed at doses up to 40 mg $cyanide.kg^{-1}$ b.w. per day, even though some of the doses were equal to or higher than the oral LD50 for cyanide. Since in the short-term studies analysed, cyanide was administered through the diet or drinking water, absence of mortality is possibly due to a slower absorption rate following dietary exposure, thus not exhausting the detoxification capacity of the enzyme rhodanese, which occurs after bolus administration in LD50 tests **(Hayes, 1967; US EPA, 2010)**. Acute oral median lethal dose (LD50) values for cyanide in laboratory animals range from 2.13 to 6 $mg.kg^{-1}$ b.w. **(EFSA, 2016)**. As a result of the occurrence of cyanide in food originating from flavouring substances, the Council of Europe **(CoE, 2000)** reviewed cyanide toxicity and established a Tolerable Daily Intake (TDI). JECFA **(FAO/WHO, 2012)** established a Provisional Maximum Tolerable Daily Intake (PMTDI) of 20 $\mu g.kg^{-1}$ b.w. of CN (cyanide) based on findings in a 13-week toxicity study on sodium cyanide conducted by the US National Toxicology Program (NTP).

CONCLUSION

Our findings indicate that intramuscular and oral application of amygdalin for two weeks did not significantly affect the haematology parameters in experimental animals. In this study, no obvious beneficial or negative effects of amygdalin administration on the blood of male rabbits were observed. Given that amygdalin is occuring cyanogenic glycoside in food with

the possible therapeutic effects and there are not many in vivo studies about its effects on biochemical and haematological parameters in blood, thus it is neccessary to be examined further.

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