COBISS: 1.08
Agris category code: L50, L70

EFFECT OF HYDROLYSABLE TANNINS ON PROLIFERATION OF SMALL INTESTINAL PORCINE AND HUMAN ENTEROCYTES

Maksimiljan BRUS [1], Tomaž LANGERHOLC [2], Dejan ŠKORJANC [3]

ABSTRACT

The aim of the study was to investigate the concentration dependent impact of chestnut and oak extracts with high concentration of hydrolysable tannins on the growth of animal and human small intestinal enterocyte cell lines.In our study, four tannin extracts from oak and sweet chestnut were used (GALLIC ACID, FARMATAN, CONTAN, TANEX). Tannins were tested on normal porcine small intestinal cell lines (PSI, IPEC-J2, CLAB), human normal (H4) and human cancerogenic (Caco-2) cell line. Proliferation of cells was followed in 96-well plate by colony count assay. In this assay, a small number of cells was initially seeded into wells and growth of cell colonies in the presence of tannins supplemented in a wide range of concentrations (0.5 μg/mL–500.0 μg/mL) was followed up to 5 days. Experiments were stopped before cell colonies started to fuse and counted using the microscope. Colony count served as an indicator for comparison between control and assay experiments. GALLIC ACID was very effective at growth inhibition of cancerogenic Caco-2 cells. Inhibition up to 20% was observed in the concentration interval 0.98 to 31.25 μg/mL. FARMATAN accelerated proliferation of IPEC-J2 and Caco-2 cell lines up to 31.25 μg/mL. CONTAN was most potent in promoting growth of IPEC-J2 in the range of 1–62.5 μg/mL. In contrast, in a range between 1–62.5 μg/mL TANEX accelerated growth of human normal cell line H4, while having little effects on other cell lines. The obtained results suggest potentially beneficial use of chestnut and oak extract in the diet of humans and animals. In lower concentrations tannins could be used for the purpose of accelerated recovery of small intestinal epithelium, but further research is needed for elucidation of the mechanisms responsible for the observed effects on cell proliferation.

Key words: hydrolysable tannins / cell growth / small intestine / human cell lines / pig cell lines

1 INTRODUCTION

Growing public concern about the use of antibiotics in livestock feed and the increasing number of antibiotic-resistant pathogens has resulted, in January 2006, in a total EU-ban on the use of antibiotics in feed for pigs. Instead of antibiotics producers started to use other feed additives to improve health and to enhance growth of domestic animals (Verstegen and Williams, 2002). In the group of plant extracts, tannins can be considered as well. Tannins as a feed additive have a high potential for livestock production, but their use is hindered particularly by the lack of knowledge about the role and impact of various tannins on animals. Tannins are a group of secondary metabolites that function as plant defense against herbivores. By chemical structure tannins can be divided into four groups (condensed tannins – proantocyaninns, hydrolysable tannins, florotaninns – brown algae and complex tannins – linked to metal or protein). Tannins, which are extracted from hardwood such as oak and chestnut trees, primarily contain hydrolysable tannins. Hydrolysable tannins possess antiviral and anti-bacterial activity, while exerting anticarcinogenic and antioxidative effects upon intestinal epithelial cells (Okuda and Ito, 2011). Hydrolysable tannins are chemically low molecular weight substances, composed primarily of gallic acid

1 University of Maribor, Faculty of Agriculture and Life Sciences, Pivola 10, 2311 Hoče, Slovenia, e-mail: maksimiljan.brus@um.si
2 Same address, e-mail: tomaz.langerholc@um.si
3 Same address, e-mail: dejan.skorjanc@um.si

as the basic unit. Different interconnections between gallic acids result in new compounds that form a group of gallotaninns. Gallic acid is related to ellagic acid, which is the basic unit for ellagitaninns. From the nutritional point of view it is important to consider the impact of individual representatives of the gallo– and ellagitaninns, as well as complex mixtures of tannins in animal feed, since synergistic or antagonistic effects between them can occur on animal health. Therefore, the aim of the study was to investigate the impact of tannins in different concentrations on the *in vitro* growth of small intestinal epithelial cell lines. In our study we used normal porcine (PSI, CLAB, IPEC-J2) and normal human (H4) cell line as well as cancerogenic colon cancer cell line (Caco-2).

2 MATERIAL AND METHODS

World unique non-cancerogenic intestinal epithelial cell lines were developed at the University of Maribor in the frame of EU-FP6 project (Pathogen Combat). Apart from human model cell line H4, porcine cell lines were developed as well (IPEC-J2 cl.1, CLAB, PSI) (Cencič and Langerholc, 2010). Cell lines were characterized for their function, expression of specific markers and they represent cells that are functionally close to the primary absorptive cells – intestinal enterocytes.

2.1 CELL LINES DESCRIPTIONS

In experiment following cell lines were used:

- Human cell lines
  - Caco-2 – were isolated from a 72-year old Caucasian male. Upon reaching confluence, the cells were found to express characteristics of enterocytic differentiation and functionality (Cencič and Langerholc, 2010).
  - H4 – normal enterocytes of immature human small intestinal foetal tissue, which is capable of tight epithelial barrier formation with transepithelial resistance (TER) and voltage (TEV) formation (Cencič and Langerholc, 2010).
- Porcine cell lines
  - IPEC-J2 – Non transformed cell line from a neonatal piglet. Enterocyte like, mucin producing cell type (Cencič and Langerholc, 2010).
  - PSI (*Pig Small Intestine*) – Pig small intestine cell line from the adult pig. Classified as crypt cell like. Forms tightly packed epithelial barrier (high TER/TEV) and weak mitochondrial ROS and NO production (Cencič and Langerholc, 2010).
  - CLAB – Cell line derived from adult pig. Mucin producing on surface. Strong response to activators with NO, ROS, and mitochondrial dehydrogenase activity (Cencič and Langerholc, 2010).

2.2 CELLS GROWTH AND INHIBITION ASSAY

All cell lines were grown in Dulbeeco`s modified Eagle`s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U/mL)/ streptomycine (100 U/mL). Cells were maintained in a humidified 37 °C incubator with 5% CO_2. Four tannin extracts from oak and sweet chestnut were used (Gallic acid, FARMATAN, CONTAN, TANEX) produced by Tanin Sevnica d.d., Sevnica, Slovenia. Concentrations of hydrolysable tannins in extracts in Gallic acid equivalent (GAE) were 20.00 mg/g GAE DW, 13.60 mg/g GAE DW, 18.03 mg/g GAE DW, respectively. Tannins were tested in a wide range of concentrations (1 µg/mL–500 µg/mL). Briefly, all cell lines were trypsinized, counted and plated in 96-well tissue culture plates in concentration 70 cells/ well (n = 5/treatment) using standard growth conditions. Depending on the cell line, cells in wells were inucubated for 5–10 days and the experiment was stopped in a point, when the colonies were clearly seen, but not yet fused to each other. Crystal violet staining was used for colony visualization and colonies were counted using light microscopy (magnification 40×). All data presented in graphs (Fig.1) were calculated as percentage of developed visible colonies in comparison to the control (cells without extract).

3 RESULTS AND DISCUSSION

Most of the scientific research of the intestine is based on experiments with tumor cell lines like Caco-2, HT29 and T84. These cell lines do not originate from small intestine, but from colonic epithelium. Anatomically, nutrient absorption takes place in the small intestine and therefore, these lines do not adequately reflect the *in vivo* situation. With regard to the cell line origin, normal cell lines used in our study were derived from the absorptive part of small intestine (jejunum) and therefore better and more reliably reflect the events in the small intestine as the before mentioned cell lines.

GALLIC ACID was very effective at growth inhibition of carcinogenic cell line Caco-2. The inhibition reached up to 20% in the concentration interval 0.98 to 31.25 µg/mL. Higher concentrations of gallic acid showed strong cytotoxicity on the used carcinogenic cell

line. Similar results on cytotoxicity have already been described (Loizzo *et al*., 2009; Yi *et al*., 2005a; Yi *et al*., 2005b). In a narrow area (1–4 µg/mL) gallic acid did not show growth inhibition of the normal human H4 line and normal porcine PSI cells. In contrast, increased cellular proliferation was observed at neonatal porcine cell line IPEC-J2 (1–2 µg/mL). From nutritional point of view this can be an important result in connection with recovery capacity of small intestine epithelium in young and also adult animals.

FARMATAN in the research demonstrated higher proliferative effect on IPEC-J2 and Caco-2 cell lines up to 31.25 µg/mL. No growth effects were observed in all other cell lines within the same concentration range. Consequently, FARMATAN supplement in piglets' diet could have wide practical use in pig production.

CONTAN significantly promoted cell growth of the line IPEC-J2 in the range between 1 – 62.5 µg/mL, as well as Caco-2 to a lesser extent. For the rest of cell lines no inhibitory effect in the same concentration interval was detected.

TANEX in a range between 1–62.5 µg/mL accelerated more efficiently the growth of human normal cell line H4 in comparison with the other cell lines. From the remaining cell lines stands out cell proliferation of PSI and IPEC-J2 cell lines, suggesting potential use of TANEX for recovery of small intestinal epithelia after disorders caused by nutritional and health stress.

4 CONCLUSIONS

The use of plant extracts in animal nutrition with a high antioxidant potential, selective antimicrobial and antiviral activity is highly desirable among animal breeders and producers of healthy food. Extracts of chestnuts and oak woods are rich in hydrolysable tannins which have been proven for their antimicrobial, antioxidative (Fernandes *et. al.,* 2009; Brus *et al.*, 2013) and antiviral effects. For the safe use of additives in feed and food, *in vitro* animal and human cell models a good and cheap alternative to animal experiments for determination of possible optimal concentration of supplementing additives (Cencič and Langerholc, 2010; Brus *et al.*, 2011).

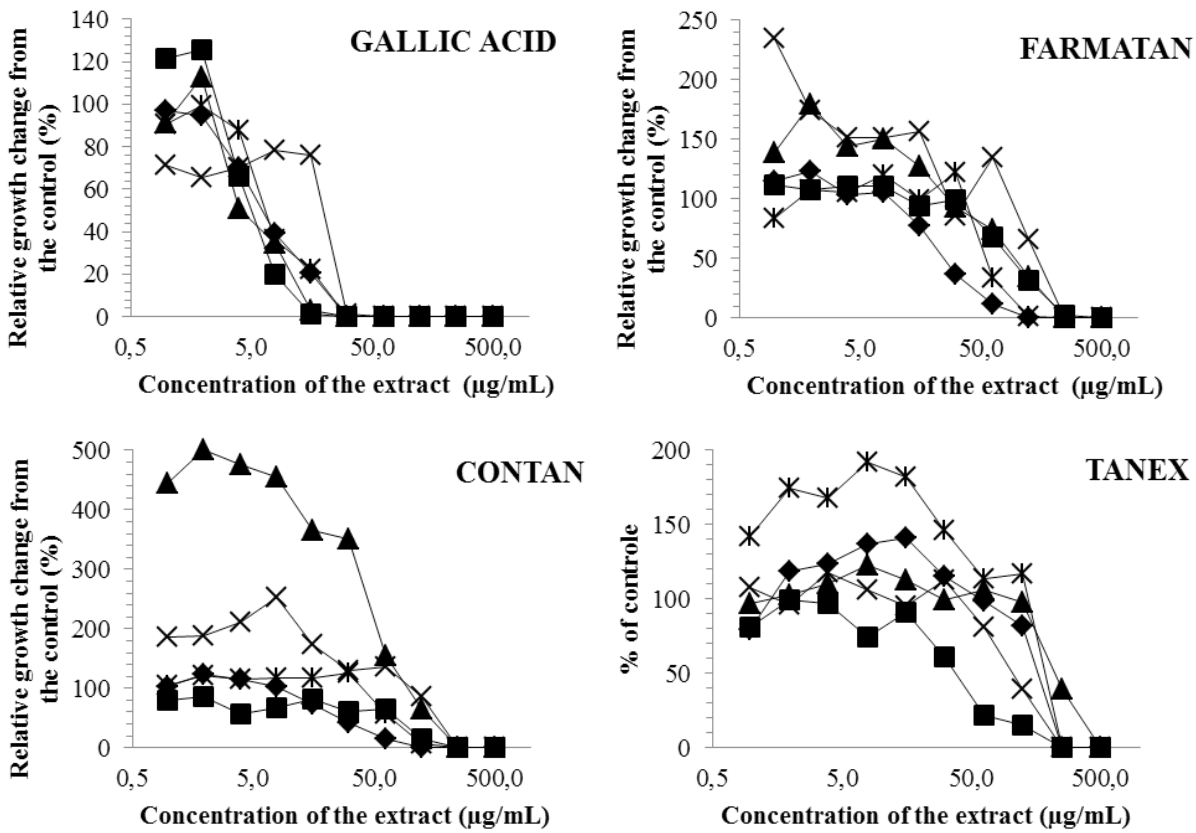

Figure 1: *Growth changes observed with cell lines in the presence of plant extracts rich in hydrolysable tannins (◆ – PSI, ■ – CLAB, ▲ – IPEC-J2, ✕ – CACO-2, ✻ – H4)*

The obtained results corroborate potential beneficial use of chestnut and oak extract in the diet of humans and animals.

5 ACKNOWLEDGEMENT

This study was financially supported by funds of Slovenian Research Agency (P1-0164 programe).

6 REFERENCES

Brus M., Langerholc T., Skorjanc D., Cencic A. 2011. Novel biological approaches to identify nutrition efficiency of feed for farm animals. Current Opinion in Biotechnology, 22, 1: S33

Brus M., Langerholc T., Skorjanc D. 2013. Intracellular antioxidative properties of hydrolysable tannins on animal and human small intestinal enterocyte cell. Current Opinion in Biotechnology, (accepted).

Cencič A., Langerholc T. 2010. Functional cell models of the gut and their applications in food microbiology — A review. International Journal of Food Microbiology, 141: S4–S14

Fernandes A., Fernandes I., Cruz L., Mateus N., Cabral M., de Freitas V. 2009. Antioxidant and Biological Properties of Bioactive Phenolic Compounds from Quercus suber L. *Journal of Agricultural and Food Chemistry*, 57, 23: 11154–11160

Loizzo M.R., Said A., Tundis R., Hawas U.W., Rashed K., Menichini F., Frega N.G., Menichini F. 2009. Antioxidant and Antiproliferative Activity of Diospyros lotus L. Extract and Isolated Compounds. *Plant foods for human nutrition*, 64, 4: 264–270

Okuda T., Ito H. 2011. Tannins of Constant Structure in Medicinal and Food Plants—Hydrolyzable Tannins and Polyphenols Related to Tannins. *Molecules*, *16*: 2191–2217

Verstegen M.W.A., Williams B.A. 2002. Alternatives to the use of antibiotics as growth promoters for monogastric animals. Conference on Antibiotic Use in Animal Agriculture, Chicage, Illinois, Oct 16–17, 2000, *Animal Biotechnology*, 13, 1: 113–127

Yi W.G., Fischer J., Akoh C.C. 2005a. Study of anticancer activities of muscadine grape phenolics in vitro. *Journal of Agricultural and Food Chemistry*, 53, 22: 8804–8812

Yi W.G., Fischer J., Krewer G., Akoh C.C. 2005b. Phenolic compounds from blueberries can inhibit colon cancer cell proliferation and induce apoptosis. *Journal of Agricultural and Food Chemistry*, 53, 18: 7320–7329