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Phytoestrogens: characterization and biological effects

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Coordinatore: Prof. Marco Esti

Tutor: Prof. Nicolò Merendino

Dottorando: Silvia Ricci

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i. ABSTRACT

In this PhD thesis, some of the biological effects of phytoestrogens, in particular of lignans and the selection and characterization of buckwheat seeds and sprouts as potential rich food sources of polyphenol and pytoestrgens are reported. The effects *in vitro* of lignans on proliferation of human colon cancer cells lines (HT29 and HCT8) showed that lignans inhibit cellular proliferation and induce arrest of cell cycle in the G1-phase on both cellular lines. In order to study the mechanism of action we observed the behaviour of cyclin D1 and p-21 proteins. The results showed that cyclin D1 decreased expression while p-21 protein level resulted increased in cells treated. Further we measured the intracellular ROS (reactive oxygen species) level in cells treated. The analysis showed a reduced concentration of ROS especially after 24 h compared to 48 h. In addition procedure for growth and characterization of buckwheat sprouts and seeds, as preliminary step for a future setting up of a proof-project for formulation of functional food rich in biologically active components like phytoestrogens was studied. Hence hydroponic culture of Buckwheat sprouts was produced and after evaluated the yields of harvest we analyzed the amount of total phenolic contents both in sprouts and in seeds. Tartary buckwheat sprouts and seeds show higher content of phenolic contents than Common buckwheat. The research produced promising results which open horizons on the possibility to produce innovative food that provide health benefit using, for the first time, powder of Tartary buckwheat sprouts.

ii. SINTESI

In questa tesi di Dottorato sono stati analizzati alcuni degli effetti biologici dei fitoestrogeni, in particolare dei lignani ed è stata effettuata la selezione e la caratterizzazione dei germogli e dei semi di grano saraceno come fonte alimentare potenzialmente ricca in polifenoli e fitoestrogeni. Gli effetti *in vitro* sulle linee cellulari tumorali di colon (HCT8 e HT29) hanno mostrato che i lignani inibiscono la proliferazione cellulare e inducono l'arresto nella fase G1 del ciclo cellulare in entrambi le linee cellulari. I risultati dell'analisi dell'espressione proteica hanno mostrato che il blocco del ciclo cellulare in G1 porta ad una diminuzione della ciclina D1 ed a un relativo aumento della proteina P21. E' stata inoltre valutata la concentrazione intracellulare dei ROS (reactive oxygen species) e si è osservato che i lignani riducono lo stress ossidativo delle cellule trattate in confronto a quelle controllo specie nei trattamenti a 24 ore. La ricerca si è poi rivolta alla selezione di semi di grano saraceno poi impiegati per la produzione di germogli da colture idroponiche e dopo l'essiccamento in stufa dei germogli, è stata valutata la resa a partire da grammi definiti di semi iniziali. E' stato poi valutato il contenuto dei polifenoli totali sia nei germogli essiccati sia nei semi. I germogli ed i semi di grano saraceno Tartary hanno mostrato un più alto contenuto di polifenoli totali in confronto al grano saraceno Common. Nel complesso questi risultati guardano vantaggiosamente all'impiego dei germogli essiccati di grano saraceno Tartary come materiale di partenza ricco in componenti bioattivi per lo sviluppo futuro di un alimento funzionale avente un effetto antiossidante ed un'attività antiproliferativa.

§ 1. INTRODUCTION

The tenet "Let food be thy medicine and medicine be thy food," espoused by Hippocrates nearly 2,500 years ago, is receiving renewed interest. In particular, there has been an explosion of consumer interest in the health enhancing role of specific foods or physiologically-active food components, so-called functional foods. Clearly, all foods are functional, as they provide taste, aroma, or nutritive value. Within the last decade, however, the term functional as it applies to food has adopted a different connotation that of providing an additional physiological benefit beyond that of meeting basic nutritional needs. The term functional foods was first introduced in Japan in the mid-1980s and refers to processed foods containing ingredients that aid specific bodily functions in addition to being nutritious. "Functional foods", "nutraceuticals" or "pharmaconutrients" are all terms, which indicate nutrients or nutrient enriched foods that can prevent or treat diseases (Hardy, 2000). These foods and food components represent the fastest growing segment in food industry. In this contest, in the last decade, cereal, buckwheat, soy, flaxseed and their products are notified the most common foodstuff based functional food and nutraceuticals (Andlauer et al., 2002). Overwhelming evidence from epidemiological, *in vivo*, *in vitro* and clinical trial data indicates that a plant-based diet can reduce the risk of chronic disease, particularly cancer. In 1992, a review of 200 epidemiological studies (Block et al., 1992) showed that cancer risk in people consuming diets high in fruits and vegetables was only one-half that in those consuming few of these foods. It is now clear that there are components in a plant-based diet other than traditional nutrients that can reduce cancer risk and cardiovascular disease, stroke, Alzheimer disease, cataracts, and some of the functional declines associated with aging. Steinmetz and Potter (1991) identified more than a dozen classes of these biologically active plant chemicals, now known as "phytochemicals." The isolation, identification and quantification of phytochemicals in foods and the evaluation of their potential benefits to human health has now become a major research topic. Actually, there are more than thousand known phytochemicals. Some of the best known phytochemicals are carotenoids (such as beta carotene, lutein, lycopene and zeaxanthin), flavonoids (such as quercetin, anthocyanins and hesperidin), limonene, indole, ellagic acid, allium, sulphoraphane, glucosilates, phenolic acids. Within the

polyphenols group are phytoestrogens and this thesis deals with the study of their biological properties (Carratù et al., 2005).

1.1 Phytoestrogens: general description

Phytoestrogens are defined by the British Working Group on Phytoestrogens of the Committee of Toxicity of Chemicals in Food, Consumer Products and the Environment of the Food Standards Agency (FSA, 2003) as any plant substance or metabolite that induces biological responses in vertebrates and can mimic or modulate the actions of endogenous oestrogens usually binding to oestrogen receptors. The majority of phytoestrogens belong to a large group of substituted phenolic compounds known as flavonoids. Three classes of flavonoid, the isoflavones, coumestans and prenylated flavonoids are phytoestrogens that possess the most potent oestrogenic activity. The major bioactive isoflavones are genistein and daidzein, which are derived from the precursors biochanin A and formononetin, respectively. Coumestrol is the most important form of coumestan consumed by humans. A class of non-flavonoid phytoestrogens, the lignans has also been identified (Figure 1). The scheme in Figure 1 may not be an exclusive list as other phytoestrogens may be identified as constituents of food in the future.

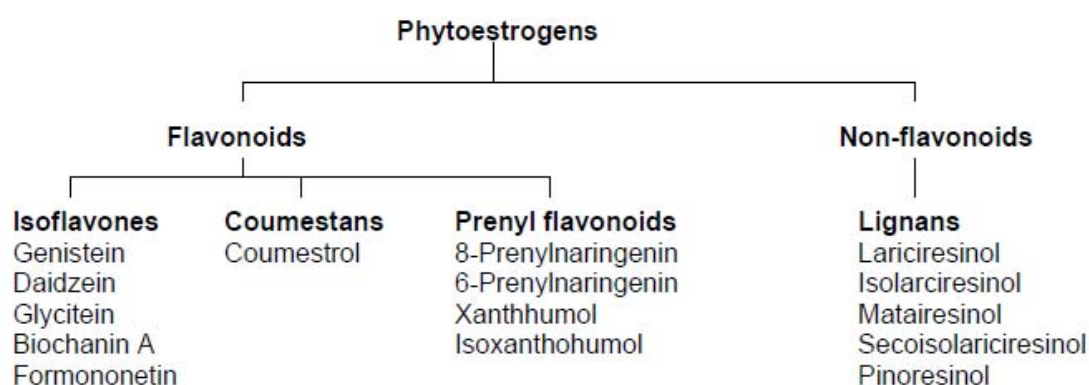


Fig. 1 Various groups of phytoestrogens and members of these groups

The phytoestrogens classes mentioned above have a similar structure to oestradiol and are able to bind the estrogen receptor (ER), preferably the ER β , although their binding affinity is lower than that of endogenous estradiol. All the structures of the

phytoestrogens possess the phenolic (bottom, left) and hydroxyl (top, right) moieties of the oestradiol structure (Figure 2) and the distances between the two groups in each compound are similar.

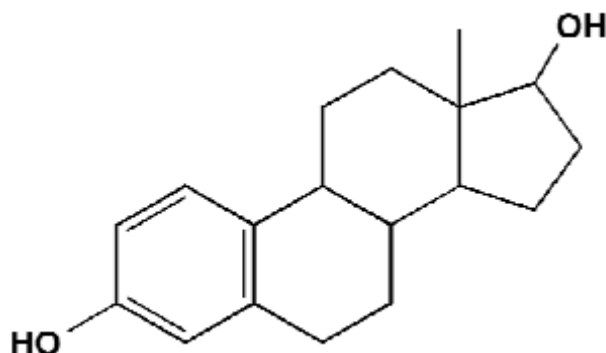


Fig. 2 Structure of oestradiol

As regards estrogenicity of the phytoestrogens, their potency is dependent on the assay used to determine the value and varies considerably. Overall, the phytoestrogens with the highest receptor binding potency is coumestrol, which is $\pm$ 10-500 times less potent than the endogenous ER β . Isoflavones are about 20,000-100,000 times less potent than ER β . Despite this weak activity, concentrations of phytoestrogens in the body are 100 to 1,000-fold higher than peak levels of endogenous estradiol in premenopausal women. In fact, the isoflavones metabolites genistein and daidzein have been shown to exert estrogenic effects even greater than endogenous estradiol at high concentrations *in vitro*, though these are outside the range of concentrations typically found in humans. Phytoestrogens show a complex mode of action via interaction with the nuclear estrogen receptor isoforms ER α and ER β , exhibiting either estrogen-agonist or estrogen-antagonist effects. Their final biological activity, assessed by cell culture assay systems, animal studies and clinical trials, depends on multiple factors such as the chemical structure of the phytoestrogens, the kind of tissue and cell type, the intrinsic estrogenic status, the route of administration, the metabolism as well as the time and the level of exposure. They are characterized by high tissue specificity and dose-dependent activity. Compounds which antagonize the estrogenic effects (antagonists) in some tissues, such as breast and uterus, while mimicking the estrogens effects (agonists) in other tissues, such as bone, brain and cardiovascular cells, are known as selective estrogen receptor modulators (SERMs). (Adlercreutz et al., 1992) (Mei et al., 2001) (Ren et

al., 2001) (Bhathena et al., 2002). The main classes of phytoestrogens and their common dietary sources are shown in Table 1 and Table 2, which suggest only the isoflavones and the lignans are commonly found in a Western diet (high in animal fats, refined grains and sugar, low in fruits and vegetables). It is possible that other phytoestrogens compounds are present in foods, which have not been detected. Until recently, most of the available information on concentrations of phytoestrogens in foods is related to isoflavone aglucones (not bound to glucose). This is due to the limitations in the analytical methods used. Data on the concentrations of isoflavone glucosides or glucones (i.e. bound to glucose), prenylated flavonoids, coumestans and lignans are more limited. Isoflavones are primarily found in legumes where they often occur as glucosides. Soybeans and soy-based foodstuffs are a particularly rich source of isoflavones, especially genistein and daidzein and to a lesser extent glycitein. Biochanin A and formononetin (which are derivatives of genistein and daidzein) are generally less prevalent in soy and are found mostly in clover and alfalfa sprouts. The coumestans of which coumestrol is the most common form, have been found in high concentrations in clover and fresh alfalfa sprouts as well. Prenylated flavonoids have been found in high concentrations in hops, which are used in some beers. The levels measured in beer are low. Note that Table 2 is a selection of published data and not a complete list of concentrations analyzed in foodstuffs. More complete lists can be found in the constructed databases, such as the United States Department of Agriculture (USDA)-Iowa State University Isoflavones Database (<http://www.nal.usda.gov/fnic/foodcomp/Data/isoflav/isoflav.html>) (Murkies et al., 1998) (Adlercreutz et al., 2002) (Benassayag et al., 2002) (Duffy et al., 2007). There is a considerable variation of phytoestrogens concentrations in different plants. The concentration of these compounds can be influenced by a number of factors including species, strain, crop year and environmental conditions. The concentrations can vary by ~2-3 fold for each factor. Processing can also alter the phytoestrogen content of foodstuffs. For example fermentation of soy into products such as tempeh, miso and bean paste reduces the isoflavone content. Cooking has also been shown to reduce phytoestrogens concentrations. However, baking or frying does not appear to alter the total isoflavones content of foodstuffs. Isoflavones and lignans are ingested mainly as glucosides and are hydrolyzed by gut bacterial and mammalian enzymes, which releases the deglycosylated compounds daidzein, genistein and glycitein (among others). These may be absorbed or further

metabolized by the gut bacteria to many specific (more potent) metabolites, including equol from daidzein and enterodiol and enterolactone from lignans. Research data suggest that once absorbed, isoflavones and lignans are extensively conjugated to glucuronides and sulfates in the liver and excreted in the bile or urine. This inhibits their ability to bind to the oestrogen receptors.

Food Item (100 g Serving)	DAI	GEN	MAT	SECO	Total ISO	Total PE
Soy products						
Soy milk (8.5 oz)	2,312.3	4,649.1	0.5	14.4	7,390.0	7,422.5
Tofu (1/4 cup)	2,988.0	5,456.1	0.3	5.8	8,677.9	8,688.0
Veggie burger (1/4 cup)	133.8	322.3	0.2	3.1	480.2	484.7
Legumes						
Hummus (1/4 cup)	0.5	5.7	9.5	1.5	8.3	605.8
Nuts and oil seeds						
Almonds (1/4 cup)	0.8	5.3	0.1	26.0	6.7	48.5
Cashews (1/4 cup)	0.5	3.5	0.1	12.8	7.5	41.5
Flaxseed (1/4 cup)	25.0	74.5	65.9	161,388.4	138.2	163,133.6
Sesame seed (1/4 cup)	0.9	0.7	41.8	2.5	3.6	2,722.8
Vegetables						
Alfalfa sprout (1/4 cup)	0.2	0.8	0.0	0.2	39.4	44.1
Broccoli (1/4 cup)	N/D	0.0	0.0	1.2	0.0	18.8
Carrots, raw (1/4 cup)	0.0	0.0	0.0	1.4	0.1	2.2
Garlic (1 tbsp)	0.9	2.4	0.8	7.2	3.5	102.6
Olives (1/4 cup)	0.5	0.7	0.0	11.9	2.4	15.4
Sweet potatoes (1/4 cup)	0.1	0.1	6.3	1.6	0.2	13.9
Tomatoes (1/4 cup)	0.0	0.1	0.0	0.5	0.2	3.9
Winter squash (1/4 cup)	0.0	0.1	0.0	4.7	0.1	39.8
Fruit						
Dried apricots (1/4 cup)	2.4	7.3	0.2	54.6	14.7	164.4
Dried dates (1/4 cup)	0.4	1.0	0.1	32.9	1.6	102.1
Cereals and breads						
Bread, multi (1 slice)	0.4	1.9	0.6	2,194.4	5.8	2,207.4
Bread, white (1 slice)	0.1	0.2	0.0	0.5	0.6	1.9
Cereal, high-fiber (1/4 cup)	0.3	0.3	0.1	3.8	0.9	9.5
Cereal, regular (1/4 cup)	0.1	0.3	0.2	1.2	0.9	2.8
Doughnuts (1 whole)	569.6	961.5	0.1	10.6	1,551.0	1,568.1
Wine, red (6 oz)	2.7	4.5	0.1	61.8	29.1	94.8
Tea, black (8.5 oz)	1.1	0.1	0.2	9.4	1.5	21.7
Tea, green (8.5 oz)	0.9	0.4	0.4	25.4	1.7	31.6

Tab. 1 Phytoestrogens content of Foods as consumed (Wet Weight) per serving (µg)

Phytoestrogens class	Examples of dietary source
Isoflavones	Legumes, lentils, chickpeas, soybean
Coumestans	young sprouting legumes and cereals
Lignans	Most cereals, linseed, fruit and vegetables
Prenylated flavonoids	Some beers (hops)

Tab. 2 Phytoestrogens and common dietary sources

As a consequence, the oestrogenically active parent compounds have relatively low concentrations in the blood. Factors influencing absorption and metabolism of phytoestrogens include diet and gut microflora. The phytoestrogens can be measured in urine, plasma, feces, semen, bile, saliva, and breast milk and the concentration of

their metabolites differ widely among individuals. In human subjects, even those on controlled diets, there is large interindividual variation in the metabolism of isoflavones and lignans, particularly in the production of equol (only 30% to 50% of adults excrete equol). This is due to gut microflora, antibiotic use, bowel disease, gender difference and concomitant dietary intake. The foods ingested with phytoestrogens can affect their bioavailability as well. Fiber intake has been shown to correlate positively with serum and urinary levels of phytoestrogens attained in women. The sum of the main phytoestrogens in plasma can reach 2000 nmol/l in people on a traditional Asian diet and 50 nmol/l in people on a standard Western diet. In effect, various studies report that isoflavones are widely consumed by Asian populations, predominantly in the form of soy. The typical concentration of genistein in soy foods is 1 to 2 mg per g of protein, and Asians consume 20 to 80 mg of genistein per day in the usual diet. By contrast, the average American ingests only 1 to 3 mg per day (Jacobson et al., 1983) (Adlercreutz et al., 1987) (Barnes et al., 1995) (Adlercreutz et al., 1997) (Ginsburg et al., 2000) (Rowland et al., 2000) (Eden et al., 2001). Breast cancer, prostate cancer, menopausal symptoms, osteoporosis and heart disease share a common epidemiology in that they are rare in South East Asian populations eating traditional diets containing soy products. In the early 1980s the possible beneficial effects of phytoestrogens in cancer prevention and other hormone-related diseases were first published. Since then literature on possible health benefits of phytoestrogens has expanded exponentially. The prevalence of symptoms of the menopause, like hot flashes, is lower in South East Asian women as compared to Western women, which may be due among others to differences in the diet. Given the demonstrated risks to conventional HRT (hormone replacement therapy), many women and their practitioners have been in search of alternatives. As a consequence, a large number of studies have been performed into the effect of soy-based products or isoflavones on menopausal symptoms. The results of these studies are inconclusive. In general, data support a benefit of soy isoflavones (30-104 mg/d) on hot flash frequency and severity, but the data are ambiguous, as positive results are often not statistically significant and strong placebo responses are observed (FSA, 2003). Kurzer (2003) mentions that only 10-20 % of the effect is due to the isoflavones per se, the rest is due to the placebo effect. Many risk factors, such as high blood pressure and diabetes are associated with cardiovascular disease. However, the underlying basis for cardiovascular disease is a combination of

atherosclerosis (excessive accumulation of lipids and smooth muscle cells in the artery) and thrombosis (development of fibrinous clots). Hormonal status is known to play a role in the development of cardiovascular disease. The similarity of phytoestrogens to oestrogens (which can decrease cholesterol levels) and the lower cardiovascular disease mortality rates in populations consuming soy suggested that phytoestrogens are protective against cardiovascular disease. Indeed, there is a considerable body of evidence to indicate that the intake of soy can have beneficial effects on low-density lipoprotein (LDL) and total cholesterol levels, but this requires that the isoflavones be consumed intact in soy protein. There is some evidence that flaxseed-fibre also has a beneficial effect on cholesterol. The effects of phytoestrogens on other factors important in the risk of cardiovascular disease such as blood pressure, thrombosis or atherosclerosis have not been extensively investigated. Osteoporosis is characterized by low bone mass and micro-architectural deterioration of bone tissues with a consequent increase in bone fragility and risk of fractures. Animal studies suggest that dietary isoflavones may exert benefits on bone mineral density and bone turnover. Epidemiological studies suggest that intakes of phytoestrogens are associated with higher bone mineral density in populations consuming relatively large amounts of soy. There have been very few intervention studies in humans in this area but results suggest small protective effects in the lumbar spine. Further studies are needed to establish whether these effects are sustained over long periods of time. Isoflavones have been shown to significantly inactivate rat and human thyroid enzymes *in vitro*, fortification of the diet with genistein did not change the thyroid function *in vivo*. So, although there is a clear effect of isoflavones on thyroid enzymes, additional factors appear necessary for isoflavones to cause overt thyroid toxicity. Data from human studies suggest that dietary soy or isoflavones are unlikely to affect the thyroid function in normal individuals with adequate iodine intake. However, for individuals with a lack of dietary iodine and/or very low thyroid activity it is possible that their thyroid function may be adversely affected by the consumption of soy-based or phytoestrogens rich foodstuffs and supplements. Also, it is possible that soy-based infant formulae may have the capacity to inhibit thyroid function in infants. However, it is not clear whether the concentrations in these formulae are high enough to realize this inhibition. It is reported that children fed soy formulae have a higher incidence of autoimmune thyroiditis but this finding may be biased because

children put on soy formula may be more likely to have autoimmune disorders, such as food allergies. Finally, limited epidemiological evidence suggests that phytoestrogens exposure is not associated with thyroid cancer risk. Oestrogens are active in the central nervous system (CNS) and are thought to influence behavior, movement and cognition (among others). They are also involved in the development and maintenance of normal immune function. Studies suggest the blood brain barrier effectively restricts phytoestrogens transfer to the CNS in adult rodents. However, despite this, relatively high dietary exposures to isoflavones in rodents have been shown to alter protein concentrations and structures in the brain as well as induce behavioral effects. The implications of these findings for humans are unclear. Studies on the effect of isoflavones on cognitive function in humans suggest small effects, but reports are conflicting. Within past few years, phytoestrogens have attracted considerable attention for their potential anticancer activity. Since almost all anticancer drugs have serious side effects, there is search for "natural" alternatives or complements to traditional therapy. Further, the increased enthusiasm in phytoestrogens as potential anticancer agents is evidenced by the published data. The population-based studies show that the mortality due to breast, ovarian, prostate, and colon cancer has a negative correlation with the phytoestrogens and cereal intake in the diet. It has been observed that the rate of breast cancer is lower in women from some populations in Asia compared with Western populations. This has led to the suggestion of a possible reverse association between breast cancer and dietary phytoestrogens intake. Studies in premenopausal women have thus focused on the potential cancer protective effects of phytoestrogens. The idea is that phytoestrogens increase the menstrual cycle length, which is thought to reduce the risk of breast cancer by decreasing the lifetime exposure of women to endogenous oestrogens. Data from studies on premenopausal women suggest that supplementation of the diet with phytoestrogens produces weak hormonal effects. However, the nature of these effects is inconsistent. The animal data on breast cancer is conflicting. A number of studies have shown that genistein has a protective effect in animal models of chemically induced cancer. However, similar experiments using (*in vitro*) tumor implant models showed that genistein stimulated the growth of established breast cancer cells. At lower concentration, they tend to stimulate the proliferation of two ER- α -dependent breast cancer cell lines whereas, at high concentration, they exert strong cytotoxic effect. In men, increased phytoestrogens consumption has been

associated with a reduced risk of prostate cancer. However, reports of hormonal effects in men from dietary phytoestrogens supplementation are inconsistent, showing no or weak hormonal effects. It has been suggested that exposure to oestrogens of phytoestrogens during development in utero, in infancy or in childhood may play an important role in the programming of hormonal homeostasis and influence the risk of developing cancer later in life. This may, in part, explain why the relatively low risk of certain cancers observed among migrant populations from South East Asia increases in subsequent generations. Long-term studies should be undertaken to establish the clinical efficacy of phytoestrogens in these conditions in humans. Due to this, there is difficulty in making wide spread recommendations about dietary intake of phytoestrogens. Thus, more research is required to establish the role of phytoestrogens in above discussed conditions. Evaluation of benefits and risks of phytoestrogens is a complex task due to interindividual variation and complexity in absorption and metabolism. Overall, it is correct to assume that consumption of phytoestrogens may be good. On the other hand, inappropriate or excessive use may be detrimental. Before making widespread recommendations for phytoestrogens intake, extensive data on specific intracellular effects, duration of exposure and disease, and results from prospective randomized studies in humans is essential. It is also necessary to determine the potential side effects of phytoestrogens. Among various phytoestrogens, isoflavones (genistein and diadzein) have been most studied. Studies on lignans are few and for coumestans very few. This might be due to lack of industrial funding and problems in analytical techniques. Study of effects of individual compounds in various clinical conditions is the need of the hour. Based on dietary phytoestrogens, structure activity relationship studies should be carried out and more synthetic and semisynthetic compounds (like ipriflavone) should be evaluated. Genetic modification of soybean and other plants and improvement in food technology to enhance phytoestrogen production is inevitable (Cassidy et al., 2000) (Chun-Sen et al., 2001) (Maggiolini et al., 2001) (Ariyo et al., 2002) (Doerge et al., 2002) (Horn-Ross et al., 2002) (Kurzer, 2003) (Cornwell et al., 2004) (Crisafulli, et al., 2004) (Dixon et al., 2004).

1.2 Lignans

1.2.1 Origin and structure

Lignans, a type of phytoestrogens, are constituents of many plants and form the building blocks for the formation of lignin in the plant cell wall. They are ubiquitous in the woody portions of plants, in plant roots, seeds, coat of seeds, the bran layer in grains, stems, leaves and fruits and they have been suggested to be involved in plant defense. Plants contain a large group of non-nutrient (poly)phenolic compounds which are synthesized to protect plants from photosynthetic stress, reactive oxygen radicals, wounds and attackers such as microbes and herbivores. Plants (poly)phenols like lignans are derived from phenylalanine units, and they have in common at least one aromatic ring structure with hydroxyl group(s). They are both derived from monolignols, *p*-coumaryl, coniferyl and sinapyl alcohols *via* their respective pathways. They are characterized by the coupling of two C₆C₃ units by a bond between positions C₈ and C_{8'}. They comprise a whole class of compounds with a similar basic skeleton, but with large variations in substitution patterns. Several hundred different lignans structures have been identified in various plants. The two most important lignans type phytoestrogens were identified as *trans*-2,3-bis(3-hydroxy-benzyl)- γ -butyrolactone (enterolactone, ENL) and 2,3-bis(3-hydroxybenzyl)butane-1,4-diol (enterodiol, END) which have been described as the major lignans present in serum, urine, bile and seminal fluids of humans and animals (Fig.3). Because these two compounds are produced in animals as opposed to plants they are usually termed the mammalian lignans to distinguish them from lignans from plants. The mammalian-derived lignans differ from plant-derived lignans in possessing phenolic hydroxy groups only in the meta-position of the aromatic rings.

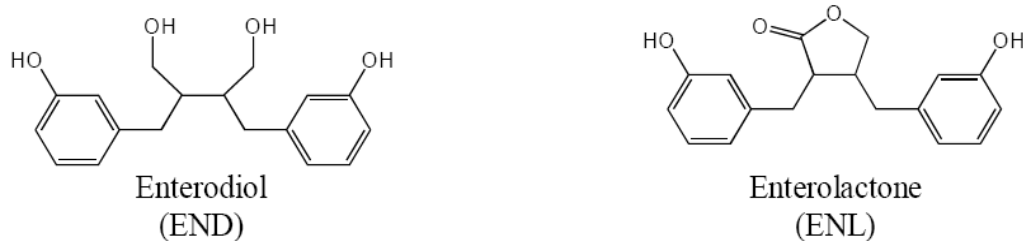


Fig. 3 Structures of lignans-type phytoestrogens END (2,3-bis(3-hydroxybenzyl)butane-1,4-diol ,enterodiol) and ENL (trans-2,3-bis(3-hydroxy-benzyl)- γ -butyrolactone, enterolactone)

After ingestion, some plant lignans can be converted to the enterolignans, enterodiol (END) and enterolactone (ENL) by the intestinal microflora and absorbed into the body. It has long been assumed that only secoisolariciresinol diglucoside (SDG), secoisolariciresinol (SECO) and matairesinol (MAT) are precursors of enterolignans, but recently it has been shown that also lariciresinol (LARI), pinoresinol (PINO) and syringaresinol (SYR) can be efficiently converted. Enterolignans are thought to be the biologically active metabolites of plant lignans (Fig. 4) (Setchell et al., 1980) (Setchell et al., 1981) (Ayres et al., 1990) (Liggins et al., 2000) (Moss et al., 2000) (Begun et al., 2004).

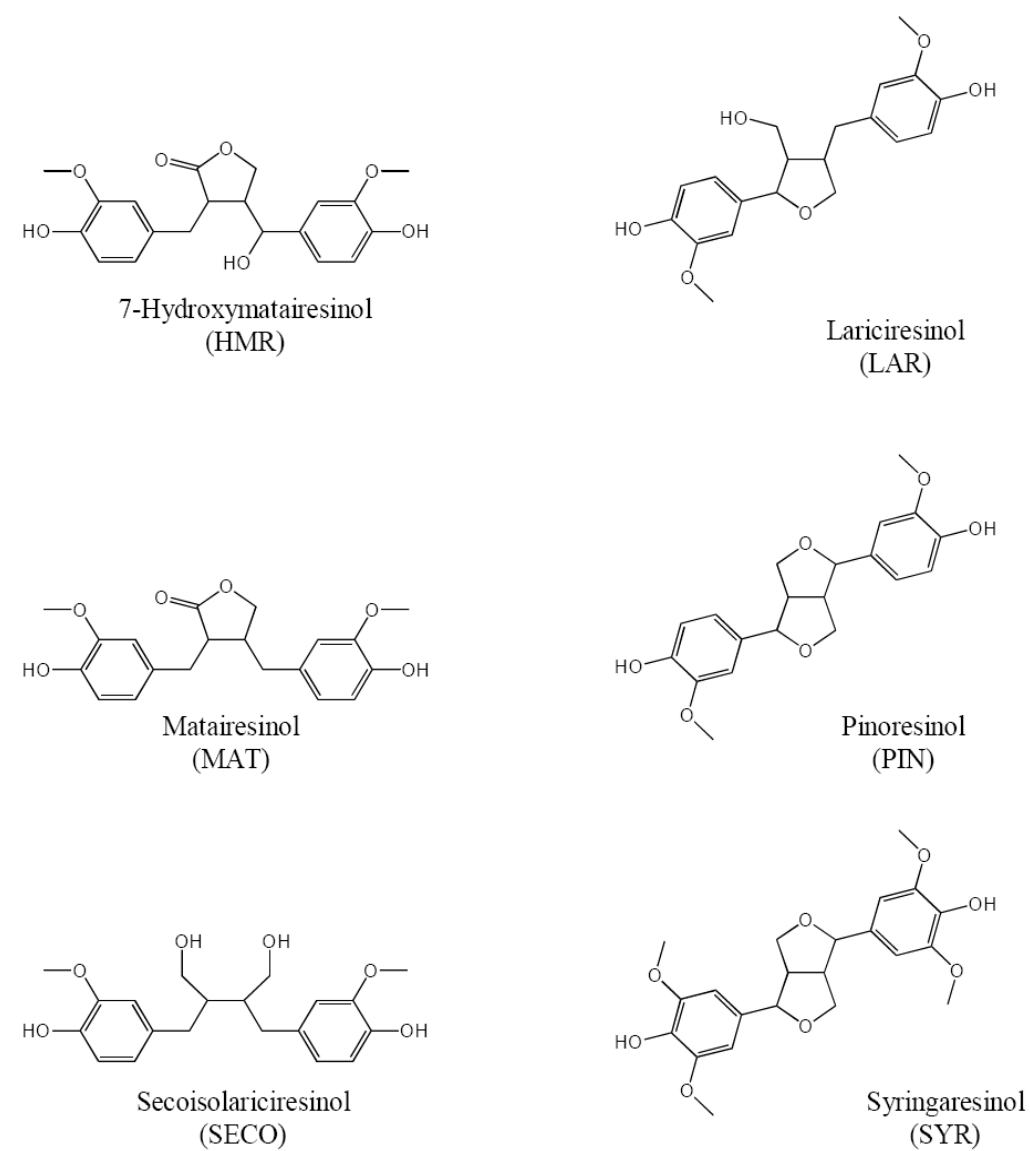


Fig. 4 Structures of plant precursors of END and ENL

1.2.2 Food sources and dietary intake

At the time of discovery of enterolignans in mammals, around 1980, the presence of lignans in plants was already known, but plant lignans had not yet been established as the dietary precursors of the enterolignans. Because urinary lignans excretion is correlated with dietary fiber intake, it was hypothesized that enterolignans precursors are present in fiber-rich foods, such as grains, nuts and legumes. The dietary origin of enterolignans could soon be confirmed, and the plant lignans SECO and MAT were identified as their precursors. Mazur et al. (1998) were the first to develop an analytical method for the quantification of these lignans in foods. In this method, lignans are released using acid and enzymatic hydrolysis, and quantified using GC-MS. Subsequently, they have reported lignans contents of a variety of Finnish foods on a dry weight basis ($\mu\text{g}/100\text{ g dry weight}$). SECO and MAT were reported to be present in several seeds and flaxseeds was identified as a rich source of SECO. In general the amount of SECO in foods was higher than that of MAT. The richest source of lignans are flaxseeds (approximately $301\text{ mg}/100\text{ g}$), which contained mainly SECO (also the amount of MAT was relatively high). In general the most commonly analyzed lignans in edible plants are SECO and MAT. Also, the lignans concentrations in sesame seeds (approximately $29\text{ mg}/100\text{ g}$, mainly pinorexinol and lariciresinol) were relatively high. For grain products, the lignin concentration ranged from 7 to $764\text{ }\mu\text{g}/100\text{ g}$. Concentrations in other oilseeds such as, sesame, clover, sunflower, caraway, poppy, and peanut were much lower. Lignans concentrations in whole grain were $48\text{-}112\text{ }\mu\text{g}/100\text{ g}$. In grain brans the concentrations were higher than in whole grain $63\text{-}299\text{ }\mu\text{g}/100\text{ g}$ whereas in flour they were lower $8\text{-}32\text{ }\mu\text{g}/100\text{ g}$. The total lignans concentrations for nuts were $96\text{-}261\text{ }\mu\text{g}/100\text{ g}$, for vegetables $16\text{-}3874\text{ }\mu\text{g}/100\text{ g}$, for fruits and berries $5\text{-}1510\text{ }\mu\text{g}/100\text{ g}$ and for legumes $0\text{-}476\text{ }\mu\text{g}/100\text{ g}$. In addition, relatively high lignans concentrations were reported for tea leaves $770\text{-}3050\text{ }\mu\text{g}/100\text{ g}$ and coffee powder $393\text{-}716\text{ }\mu\text{g}/100\text{ g}$. The total lignans content of cereals species can be in the following order: rye > wheat > oat > spelt wheat > Japanese rice > wild rice > buckwheat > barley > amaranth > corn > millet > red rice > brown rice. Lignans contents in beverages ranged from 0 for cola to $91\text{ }\mu\text{g}/100\text{ ml}$ for red wine. Although the difference of lignans content maybe at least partly due to difference in analytical methods, it is also possible that the content of some lignans may vary within the same species depending on factors such as genetic factors or

growth conditions. Studies report that lignans values in boiled vegetables were on average 25% lower than those in raw vegetables, whereas after frying, lignans concentrations were on average 30% higher. The increased lignans concentrations after frying can be explained mainly by the decreased moisture content of the fried foods. On a dry weight basis, the amount of lignans after frying decreased with 25%, comparable to what it saw for boiling. The vegetables were fried in margarine, which we have shown also to contain some lignans. However, it was calculated that the maximum contribution from lignans in the margarine was less than 1%. The effects of food preparation on lignans contents have only been reported for baking of bread, thermal treatments of olive oil and roasting of pumpkin seeds. Muir & Westcott (2000) reported that SECO diglucoside (SDG), purified or as flaxseeds, added to wheat flour before the preparation of bread, was stable in the bread making process. Besides, they found that SDG could withstand the higher temperatures in the core during baking. Brenes et al. found that microwave heating of olive oil for 10 min did not change the amount of lignans. Even after 25 h (simulated) frying at 180°C, only 20-50% of the lignans were lost, whereas other phenolics compounds were almost completely destroyed. When olive oil was boiled with water (at pH 4-6) for 30 min, a large proportion of lignans leached into the water phase, but the total decrease in the lignans concentration was only 30%, irrespective of the pH. Murkovic et al. (2004) reported that SECO in pumpkin seeds was completely destroyed after 20 min of roasting. Thus, a further evaluation of the effects of food processing might increase the reliability of lignans intake estimations (Mazur et al., 1996) (Ross Barcelo, 1997) (Mazur et al., 1998) (Mazur et al., 1998a) (Liggins et al., 2000) (Muir et al., 2000) (Heinonen et al., 2001) (Brenes et al., 2002) (Cornwell et al., 2004) (Dixon et al., 2004) (Murkovic et al., 2004) (Penalvo et al., 2004) (Branca et al., 2005) (Penalvo et al., 2005) (Raffaelli et al., 2006) (Smeds et al., 2007).

1.2.3 Bioavailability and metabolism

Consumption of lignans and subsequent exposure to enzymatic and bacterial activity in the mouth, stomach, intestines, colon and cecal generates lignans metabolites (Fig. 5) discovered in human urine already in the 1980s. In foods, lignans occur as glycosidic conjugates, which are hydrolysed by bacterial β -glucosidases in the gut and then released as aglycones (Fig.6, Fig.7). When these foods are consumed, the chewing action of the mouth physically breaks down lignans into small particles. The first step of metabolism may involve removal of the attached sugars in the lignans glycosides a reaction catalyzed by glycosidase. Glycosidase activities can occur in the food itself (endogenous or added during processing) or in the cells of the gastrointestinal mucosa or can be secreted by the facultative anaerobic colon microflora. Colon microflora is also important in lignans fermentation process that occurs in the distal end of the digestive system. Gut microflora can metabolize plant lignans further into mammalian lignans (END and ENL) and enterodiols can be further oxidized to enterolactone. The importance of microflora in the metabolism of lignans has been demonstrated both in germ-free animals and in humans by the administration of antibiotics, which prevented the production of mammalian lignans. *In vitro* studies have also demonstrated the efficient production of mammalian lignans from the dietary precursors by human fecal flora. These suggest that the primary site of their production is the cecum and colon. Two bacterial strains, *Peptostreptococcus* sp. Strain SDG-1 and *Eubacterium* sp. Strain SDG-2, capable of demethylation and dehydroxylation, respectively, were isolated from a human fecal suspension. These findings further suggest that the formation of END and ENL is not the result of spontaneous chemical reactions but due to the metabolic reaction of viable intestinal bacteria under anaerobic conditions. *In vitro* and *in vivo* experiments have revealed the plant precursors of the mammalian lignans enterodiol and enterolactone to be secoisolariciresinol, matairesinol, lariciresinol, cyclolariciresinol (isolariciresinol), pinoresinol, syringaresinol, 7-hydroxymatairesinol, arctigenin and its glycoside arctiin. In addition, 7-hydroxyenterolactone and enterofuran have been identified as mammalian lignin metabolites in human urine. An *in vitro* study showed that 7-hydroxyenterolactone was a metabolite of 7-hydroxymatairesinol, and enterofuran a metabolite of lariciresinol, pinoresinol and secoisolariciresinol. Some differences in proportions of different lignin metabolites emerge when comparing

results from *in vitro* and *in vivo* experiments. For example, 7-hydroxymatairesinol was converted to both 7-hydroxyenterolactone and enterolactone in an *in vitro* incubation with faecal flora, whereas orally administered 7-hydroxymatairesinol was mainly metabolized to enterolactone in rats. The fermentation of dibenzylbutane and methylenedioxybridged furanofuran lignans has been found to consist of 4 steps: deglucosylation, demethylation, dehydroxylation and dehydrogenation (Stitch et al., 1980) (Axelson et al., 1981) (Setchell et al., 1981) (Borriello et al., 1985) (Liggins et al., 2000) (Heinonen et al., 2001) (Saarinen et al., 2002) (Saarinen et al., 2002a) (Wang et al., 2002). Absorption of lignans occurs as aglycones. Wood-originated lignans are already in unconjugated form and thus can be absorbed in the upper parts of the small intestine. Lignans can be reconstituted in the intestinal epithelium during absorption or in the liver by UDP-glucuronosyltransferases and sulphotransferases.

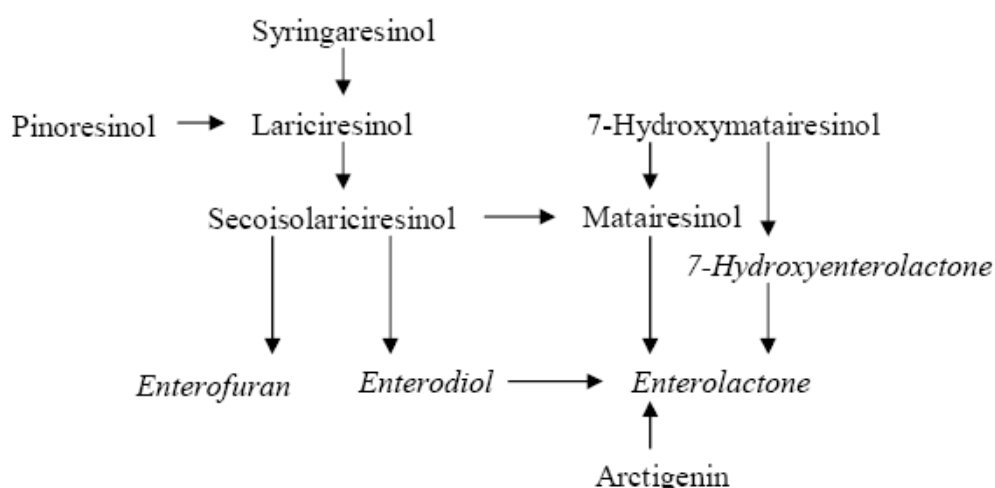


Fig. 5 Metabolic pathways of plant lignans to the mammalian lignans enterofuran, enterodiol, enterolactone and 7-hydroxyenterolactone

Additional metabolism beyond glucuronidation or sulphonation may also occur in the liver; enterolactone and enterodiol with extra hydroxyl groups have been identified in human urine after flaxseeds ingestion. Oxidative metabolism has been suggested to be a means of disposing of lignans from the mammalian body. Plasma lignans circulate either as glucuronide and sulphate conjugates or as free forms. Lignans and excess unabsorbed lignans metabolites, are excreted in the urine and *via* bile into fecal matter. ENL and END are excreted in the urine mainly as glucuronides, sulphonates, and to a minor extent as free aglycones (Adlercreutz et al., 1995) (Jansen et al., 2005).

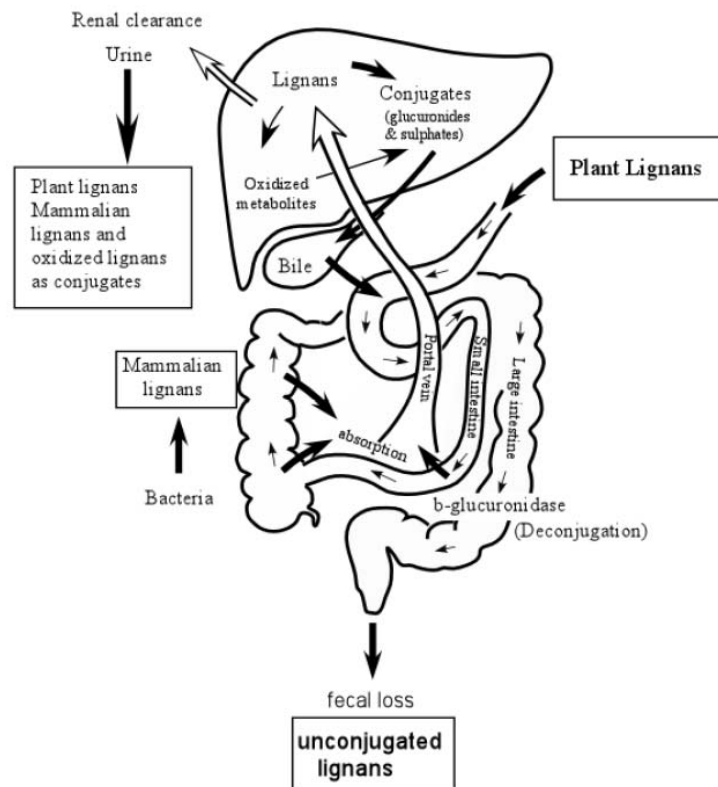


Fig. 6 Enterohepatic circulation of plant lignans

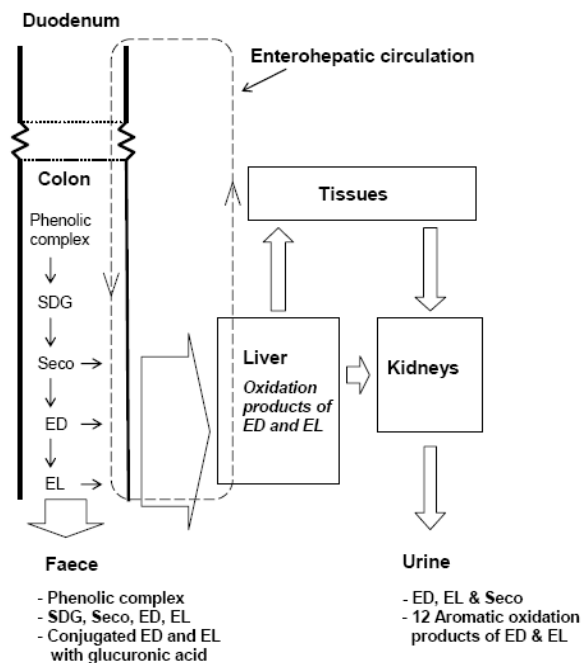


Fig. 7 Intestinal conversion of secoisolariciresinol diglycoside (SDG) from phenolic complex and absorption and excretion of SECO, END and ENL.

Conjugated lignans, which are excreted through bile, undergo enterohepatic recycling by hepatic phase II enzymes (lignans are re-excreted *via* the bile duct into the intestinal tract, deconjugated by the bacterial β -glucuronidases and sulphatases, and reabsorbed by the intestinal cells) or they are flushed away in feces in free form. Most of the lignans are eventually excreted *via* feces and urine in mammalian organisms. A study reported that after ingestion of secoisolariciresinol diglycoside, over 50% of the lignans were excreted in feces and 30% were present in urine. In tissues, the greatest concentrations were in those tissues involved in lignans metabolism, like intestinal, hepatic, and renal tissues and in blood. Moreover, some lignans were found in the uterus. The presence of lignans in human semen, saliva, breast aspirate or cyst fluid, prostate fluid and amniotic fluid has also been reported. In humans, average levels of ENL in serum/plasma are usually less than 10-30 nmol/l. However, values up to 1000 nM have been reported for specific groups, such as vegetarians, and after intervention with lignans-rich products. Pharmacokinetics relates to the rate of availability and elimination of mammalian lignans from different organs within the body; inter-individual variation is large and in individuals consuming lignan-containing foods, serum/plasma ENL levels may be considerably over 100 nmol/l. High concentration of metabolite lignans therefore, might be achievable in the enterocytes of the gut lumen where lignans are hydrolyzed by intestinal flora and then absorbed (range μ M). Various studies reported big intraindividual variations, affected by a range of different factors such variation in the microbiota, intestinal transit time, structures of the lignans and composition of the diet and food matrix. Serum ENL concentrations and a single measurement of ENL is insufficient to estimate the basal level in human subjects. In effect, the stereochemical structure of SDG and SECO has been shown to determine the chirality and the composition pattern of END and ENL and their oxidation products. Further crushing or grinding of whole food matrix has been shown to increase the levels of plasma END and ENL in humans compared with whole food. In *in vitro* fermentation models, the formation of END and ENL is increased by high amounts of carbohydrates, dietary fibre and xylanase treated rye bran. An increase in fat content in the diet decreases the urinary excretion of lignans in both rats and humans. (Axelson et al., 1981) (Adlercreutz et al., 1987) (Bannwart et al., 1989) (Rickard et al., 1998) (Jacobs et al., 1999) (Cassidy et al., 2000) (Muir et al., 2000) (Rowland et al., 2000) (Horn-Ross et al., 2002) (Scalbert et al., 2002) (Wang et al., 2002)

(Kilkkinen et al., 2004) (Stattin et al., 2004) (Aura et al., 2005) (Hongyan et al., 2005) (Kuijsten et al., 2005) (Walcott et al., 2005).

1.2.4 Biological activities and health effects

Lignans possess a range of biological activities *in vivo* and *in vitro* systems, including antioxidant, antitumor, weakly estrogenic, and anti-estrogenic properties, and inhibition of enzymes involved in the metabolism of sex hormones. The protective effects of mammalian lignans may be due to their ability to compete with E2 for the type II estrogen receptor, to inhibit enzymes involved in the metabolism of sex hormones and act as antioxidant. Lignans may affect enzymes involved in the formation of estrogens, such as aromatase, and 5 α -reductase, 17 β -hydroxysteroid dehydrogenase and may to enhance the synthesis of sex hormone binding globulin (SHBG) which may subsequently to modulate the binding of sex hormones (free estradiol). For example, ENL has been proposed to affect sex hormone production *in vitro* by inhibiting the action of steroid-metabolizing enzymes such as aromatase, an enzyme converting testosterone and androstenedione to 17 β -oestradiol and oestrone, respectively. An ER independent pathway may be involved as well. Human studies have demonstrated non-consistent effects of flaxseeds on endogenous sex hormone production and metabolism. A study reported no change in plasma total or free testosterone levels or SHBG level in men consuming 13.5 grams of flaxseed per day for six weeks. Three studies with postmenopausal women reported that consumption of flaxseed changed sex hormone levels in the urine or serum (Saarinen et al., 2002a) (Shigang et al., 2008). Most of lignans containing hydroxyl group was suggested to exhibit antioxidant action mechanisms according to the number or position of hydroxyl group especially in cells not expressing estrogen receptors. In Trolox-equivalent antioxidant activity (TEAC) and chemiluminescence (CL) assays, 8-hydroxypinoresinol glycoside and 8-hydroxypinoresinol showed high antioxidant properties. The aglycone hydroxypinoresinol displayed more powerful antioxidant activity than pinoresinol. Likewise, aglycone 9- hydroxypinoresinol was more potent than its precursor, petasignolide A. Thus, the antioxidant action of pinoresinol derivatives depends on the number of hydroxyl group in the structure. The antioxidative function of sesamin on exercise-induced lipid peroxidation was observed in animals using strenuous physical exercise as a trigger for oxidative stress. Sesamin, scavenging free radicals, exerted a strong protective effect against

exercise induced lipid peroxidation. Separately, syringaresinol and sesamin, isolated from Chinese propolis, were observed to inhibit lipid peroxidation in rat liver microsomes potently. Consistent with this, sesamin exhibited an antioxidative effect on lipid and alcohol metabolism in the rat liver. Further, sesamin and sesaminol elevated tocopherol concentration and decreased thiobarbituric acidreactive substance (TBARS) level in the blood plasma and liver of rats. In a separate experiment, sesamin was more effective than sesamolin in reducing serum and liver lipid levels while sesamolin is stronger in increasing hepatic fatty acid oxidation. The antioxidant activity of some plant lignans and of the enterolignans has been evaluated in several *in vitro* test systems at concentrations ranging from 10 100 μ M. They did not show to have prooxidant activity in this concentration range. In the FRAP (Ferric Reducing Antioxidant Power) assay, SECO and MAT had high antioxidant activity compared to ascorbic acid. Some lignans, with antioxidant activity, were observed to express a neuroprotective action in excitotoxin-induced neurotoxicity in rat cortical or hypoxic neuronal cells. Furthermore, the antioxidant action of lignans from edible plants was extended to their neuroprotective action in animal experiments. Oral administration of 9-hydroxypinoresinol and its glycoside, petasalignolde A, showed a protective effect on the seizure and mortality caused by kainic acid. In addition, these lignans successfully prevented the loss of the GSH peroxidase activity and the lipid peroxidation in brain tissue, which was exposed to kainic acid, an excitotoxin. In comparison, 9-hydroxypinoresinol, a metabolite of petsalignolide A, was more effective than its precursor glycoside, petasalignolde A in preventing kainic acid induced neurotoxicity. Under the same condition, quercetin or pinoresinol, despite their antioxidant action, showed no significant effect on the seizure and mortality caused by kainic acid. Thus, peatsalignolide A and its aglycone, 9 hydroxypinoresinol seems to have antioxidant activity in brain tissue, and thereby exert a neuroprotective effect. Thus, the extract containing 9- hydroxypinoresinol derivative may be usefully used in the prevention and treatment of neurodegenerative diseases. Taken together, antioxidant action of lignans is supposed to be responsible for various bioactivities of lignans, since cellular oxidative stress is intimately linked to disease states such as carcinogenesis, inflammation or atherosclerosis. However, other bioactivities of lignans are not necessarily related to the number of hydroxyl group, suggesting that the antioxidant action may not be necessarily required to the expression of various bioactivities (Setchell et al., 1981) (Adlercreutz et al., 1987)

(Welshons et al., 1987) (Ruiz-Larrea et al., 1997) (Andreasen et al., 2001) (Hou et al., 2003) (Ghafoorunissa et al., 2004) (Ikeda et al., 2004) (Kiso et al., 2004) (Eklund et al., 2005) (Hu et al., 2007). The structure of lignans, as phytoestrogens, is similar to that of endogenous estrogens, such as 17- β estradiol. Both enterolignans and plant lignans may bind to the estrogen receptors α and β , but at low affinity compared to endogenous estrogens. They may act as a weak estrogen agonist or antagonist depending on the endogenous estrogen concentration. There is considerable evidence from epidemiological studies that correlate high concentrations of lignans in body fluids, due to dietary intake, with a low incidence of hormone-dependent tumors (breast, ovary and prostate) and non hormone-dependent cancer (colon) (Heinonen et al., 2001). Various *in vitro* experiments suggested ENL and END significantly inhibited the growth of human colon tumor cells and the E2 (17- β -estradiol) induced proliferation of MCF-7 breast cancer cells was inhibited by ENL. In human breast cancer cell line (MCF 7), ENL at 10 nM significantly inhibited the growth of cells. At a lower dose (0.5-2 nM), the effect was stimulatory for cell proliferation; the dose amount used is the same as the levels of estrogen hormone estradiol circulating under normal conditions (1 nM). This and other studies suggested that ENL is agonist towards estradiol receptors in stimulated MCF-7 breast cancer cells at a low dose but antagonist at higher doses, hence indicating a possible mechanism by which it affects growth of estrogen sensitive cells. In prostate cancer cell lines (PC-3, DU-145, LNCAP), 10-100 μ M ENL and END significantly inhibit growth of all cell lines. Mammalian lignans can stimulate proliferation and DNA/protein synthesis of breast cancer cell lines at 1-10 mM concentrations (low oestrogenic activity). *In vivo* they show to reduce epithelial-cell proliferation and the number of aberrant crypt foci in various animal models (Wang et al., 1997) (Kitts et al., 1999). High ENL and END concentrations can also inhibit the growth of ER positive and ER-negative breast cancer cells. The interplay of lignans and ER α/β might result in competition with endogenous oestrogens, thus modulating the biological activity of oestrogens in target tissues. Interestingly, women with breast cancer or with a high risk for breast cancer excrete lower amounts of lignans in urine compared to healthy women they have the same diet. Anticancer effects of SDG, ENL, END and 7-hydroxymatairesinol may, in part, be associated with their antioxidant capacities, as observed *in vitro* assays. In most studies, plant and mammalian lignan concentrations were at micromolar levels, which can be over one hundred/thousand-fold the

concentrations generally found in the plasma of human subjects. Although the action of lignans action has been postulated to occur *via* ERs, conclusive evidence for oestrogen-like activity *in vivo* has not been found. Early studies with ENL showed no clear oestrogenic effects on uterine weight or uterine RNA synthesis in female mice and rats. In the latter study, ENL inhibited oestrogen-stimulated RNA synthesis in the rat uterine only when administered 22 hours before oestradiol. Saarinen *et al.* subsequently reported that ENL, 7-hydroxymatairesinol, MAT and SECO had no oestrogenic/antioestrogenic activity or aromatase-inhibiting capacity in immature rats, as judged by uterine growth. Furthermore, END and ENL at concentrations < 1 mM did not show any activation of a reporter gene *via* α - and β type oestrogen receptors. In these studies, the mechanism of the action of lignans in DMBA-induced mammary tumours remained partly unknown. Several studies with young and adult rats have shown that flaxseed supplementation, pure SDG and nortrachelogenin may cause some oestrogen-like effects in rats, but the effect is dependent on the life stage. Thus, short-term experiments with flaxseeds have yielded no conclusive evidence for oestrogenic action of lignans. Similarly, no clear changes in the metabolism of sex hormones or SHBG, which has suggested to be in part responsible for lowering the risk of hormone-dependent cancers, were detected. Besides the estrogenic and antioxidant activities of lignans, there are several other mechanisms, which might explain the health effects of lignans. Some examples of other mechanisms are interaction with other receptors like the PXR-receptor, inhibition of enzyme expression, for example the enzymes involved in blood pressure regulation, or inhibition of particular enzyme activity. However, because these mechanisms are not extensively studied, they are not discussed further (Sung et al., 1998) (Thompson et al., 1998) (Lee et al., 2004) (Jacobs et al., 2005) (Thompson et al., 2005) (Sok et al., 2006) (Cui et al., 2007). There has been increasing interest in phytoestrogens due to a wider awareness of their possible beneficial effects on human health included the modulation of immune system. Actually little is known about the impact and the mechanism of lignans and other phytoestrogens on the immune system. From *in vitro* models it is know that isoflavone genestein dose-dependently affects lytic activity of NK cells and inhibits cell proliferation as well as the production of cytokines, leukotrienes and oxide nitric (Prasad K, et al. 1991). Recently, the carrageenan-induced rat paw oedema formation has been frequently employed in the screening of anti-inflammatory agents. LAR and isolariciresinol expressed their anti-

inflammatory activities by significantly inhibiting carrageenan-induced hind paw edema in mice. These were supported by their potent in vitro inhibitory effect on the production of TNF- α , a proinflammatory cytokine. Lariciresinol glycoside, pinoresinol, pinoresinol glycoside and syringaresinol glycoside also showed anti-inflammatory effects. Macrophages and lymphocytes, playing an important role in host immune responses, are proliferated and activated by inflammatory signal compounds, such as lipopolysaccharide (LPS). As a result, they secrete proinflammatory mediators such as cytokines (TNF- α , ILs) and lipid mediators (prostaglandin E and leukotriene B), as well as reactive oxygen and nitrogen intermediates. Isolariciresinol, lariciresinol glycoside, pinoresinol, pinoresinol glycoside and syringaresinol glycoside were found to significantly inhibit TNF- α production from mouse macrophages. In addition, pinoresinol and syringaresinol glycoside showed significant suppressive effects on NO production triggered by LPS. However, lignin compounds seemed to interfere with biosynthetic pathway for TNF- α production, rather than NO formation, in activated macrophages. In related experiment to see the effect of lignan on concanavalin A or interleukin-2 induced lymphocyte proliferation, syringaresinol glycoside potently inhibited T lymphocyte proliferation induced by concanavalin A or interleukin-2. In addition, pinoresinol showed a significant inhibitory effect on cytokine production from LPS (or phytohemagglutinin)-stimulated human peripheral mononuclear cells (Prasad et al., 1997) (Cho et al., 2001) (Küpeli et al., 2003) (Cicala et al., 2007) (Sandra et al., 2008). Lignans complex from flax seed was suggested be beneficial in preventing atherosclerosis and reducing risk factors for coronary artery disease and stroke. Concerned with this, there are epidemiological studies on the associations between enterolignan concentrations in biological fluids or the intake of plant lignans and chronic disease risk. In case control studies, there was an inverse associations of serum lignans with cardiovascular diseases in Finnish studies. The lignans SDG from flaxseeds has been shown to be effective in decreasing serum cholesterol and reducing the extent of atherosclerosis in the hypercholesterolemic rabbit. Additionally, flaxseed lignans SDG was suggested to prevent and alleviate hypercholesterolaemic atherosclerosis by inducing adiponectin mRNA expression and showing beneficial effects on lipid metabolism in diet-induced obesity in mice. Nonetheless, it was reported that flax lignans complex failed to produce regression of atherosclerosis (Prasad et al., 2000). SDG was shown to reduce total serum

cholesterol in rabbits and it had antihypertensive and angiogenic activity in rats. In several human intervention studies, consumption of flaxseed could reduce total and LDL cholesterol, without an influence on HDL or total triglycerides. However, besides lignans flaxseed also contains relatively high amounts of α -linolenic acid and soluble fiber, so it is not clear whether these results can be attributed to the lignans present in flaxseed (Ward, 1993) (Vanharanta et al., 1999) (Heinonen et al., 2001) (Vanharanta et al., 2003). Diabetes mellitus, a disorder caused by defects in insulin secretion, sensitivity, or both, is characterized by hyperglycaemia and it is followed by different complications in the vascular system and in some tissues and organs. In experimental animal models of diabetes, a preventive or delaying effect of lignans on the development of diabetes mellitus has been obtained. A few studies on glucose metabolism have been performed with flaxseed or the phenolic complex. Postmenopausal women ($n=25$) with hypercholesterolaemia given food rich in lignans like flaxseed showed reduced glucose and insulin levels. In humans with hypercholesterolaemia, the phenolic complex had a reducing effect on fasting plasma glucose levels. In another human intervention study using type 2 diabetic hypercholesterolaemic postmenopausal women ($n=30$), the subjects showed modest improvements in long term glycaemic control, measured as reduction in glycosylated haemoglobin, after eating lower amounts of the phenolic complex for eight weeks, but there was no effect on fasting glucose and insulin sensitivity. Another flaxseeds component, the mucilage given to young healthy humans, has previously been shown to reduce postprandial glucose levels in blood plasma. These studies indicate that SDG and/or other component/s in flaxseeds might have lowering effects on glucose levels. Flaxseeds and SDG are suggested to protect against renal diseases. Renal function in animal models or in humans has been shown to improve with flaxseeds treatment or SDG (Cunnane et al., 1993) (Clark et al., 2000) (Prasad et al., 2001) (Lemay et al., 2002) (Velasquez et al., 2003) (Bouché et al., 2004) (Prasad et al., 2005) (Prasad et al., 2007) (Zhang et al., 2007).

§ 2. COLORECTAL CANCER

“Colorectal cancer is a cancer that forms either in the tissues of the colon, the longest part of the large intestine, or in the tissues of the rectum, the last part of the large intestine before the anus” (definition taken from National Cancer Institute, U.S.). Colorectal cancer is the third most common cancer (9.4%) worldwide after lung and breast cancers. It ranks fourth in mortality (7.9%), after lung, stomach and liver cancers. Colorectal cancer ranks among the three most common cancers in terms of both cancer incidence and cancer-related deaths in most Western industrialized countries, every year nearly one million people worldwide develop colorectal cancer. Lifetime risk of colorectal cancer may reach 6% of the population in the Western industrialized countries. Incidence rates of colorectal cancer in countries of the European Union (EU) according to the 2006 estimates varied by a factor of 3 for men and a factor of 2 for women, with the lowest age standardized incidence rates to be observed in Greece (31/100.000 for men and 21,3/100.000 for women) and the highest incidence rates to be observed in Hungary (106/100.000 for men and 50,6/100.000 for women). Incidence rates estimates for the EU are 59/100.000 for men and 35,6/100.000 for women. The age-specific incidence of colorectal cancer rises sharply after 35 years of age, with approximately 90% of cancers occurring in persons older than 50 years, for this individuals aged from 50 to 74 years old are invited every two years for bowel screening. In Italy the number of deaths from colorectal cancer represents about 11% of the cancer mortality in men and 14% of the cancer mortality in women. The most recent data from Italian population-based cancer registries show a relevant variation in colorectal cancer burden by sex and geographical area. Colorectal cancers are the third most prevalent cancers in the United States and accounts for 10% of cancer deaths in both men and women. Large variations in incidence rates were observed with the lowest incidence rate to be observed in Africa (incidence rate in middle Africa: 2,3/100.000 for men and 3,3/100.000 for women) and the highest to be observed in Australia, North America and Europe (highest incidence rate in Australia / N. Zealand: 48,2/100.000 for men and 36,9/100.000 for women). There is no clear trend in global age standardized incidence rates of colorectal cancer. In countries of relatively low-income economy, which have recently made a transition to a higher-income economy (e.g. eastern and southern European countries, Japan, Singapore), a rapid increase in incidence rates

has been observed. Migrant studies, where populations migrate from low-risk to high risk areas, have demonstrated that the colorectal cancer incidence among the immigrants quickly (within one generation) approach the incidence of the native population of the host country with the largest increase occurring in risk of cancer in the distal colon. The large international variation in incidence rates and the shift in sub-site distribution (proximal or distal segment of colon) after migration, indicate the importance of environmental factors and life style factors as a part of colorectal carcinogenesis. Lifestyle factors including diet, overweight, low physical activity and smoking may account for 70% of colorectal cancers. Diet is a major but controllable factor that affects colorectal carcinogenesis. Other important factors are alcohol intake, non steroidal anti-inflammatory drugs (NSAIDs) intake and hormone replacement therapy (HRT) in post-menopausal women (the two last are associated with a reduced risk of colorectal cancer) (Schottenfeld et al., 1996) (Parkin et al., 2002) (Stewart et al., 2003) (Crocetti et al., 2004) (Ferlay et al., 2004) (Weitz et al., 2005) (Grande et al., 2007) (Verdecchia et al., 2007). Age, personal history of previous colorectal cancer or adenomatous polyps, family history of colorectal cancer, chronic bowel inflammatory disease, and presence of either HNPCC (Hereditary Non-Polyposis Colorectal Cancer or Lynch syndrome) or FAP (Familial Adenomatous Polyposis) are considered as established risk factors of colorectal cancer to. According to the American Cancer Society, individuals that have a personal history of colorectal cancer, a personal history of adenomatous polyps and have a family history of colorectal cancer, are at increased risk of developing colorectal cancer. Germline mutations in the mismatch repair genes, including *MLH1*, *MSH2* and *MSH6*, cause Lynch syndrome, and the syndromes' penetrance is approximately 80% for colorectal cancer. Microsatellite instability was described as a hallmark of Lynch syndrome. In addition have a history of inflammatory bowel disease (including ulcerative colitis and Crohn's disease) of significant duration or have one of the two hereditary syndromes (HNPCC or FAP), are at high risk of developing colorectal cancer. For individuals at increased and high colorectal cancer risk screening and surveillance techniques should be provided to decrease incidence and mortality rates. Adenomatous polyps are neoplastic benign epithelial tumours and most adenocarcinomas of the colon and rectum arise from pre-existing adenomatous polyps via the adenoma–carcinoma sequence. Most cancer-causing mutations occur in somatic cells, and sporadic colorectal carcinomas arise from a

multistage process in which epithelial cells harbour multiple genetic changes in tumour-suppressor genes and proto-oncogenes. Some of the genetic changes underlying intestinal tumour progression are summarized in Figure 8. The adenomacarcinoma sequence is widely accepted to be one of the main pathways, and the *APC* tumour-suppressor gene to be one of the first genes mutated during intestinal neoplasia. The *APC* gene is mutated in 80% of sporadic colon cancers and in all patients with FAP, and is followed by mutation or loss of other tumour suppressor genes (*p53* and *SMAD2/4*) and mutation of the *KRAS* protooncogene. In addition to a mutation in one allele, inactivation of tumour-suppressor genes requires losses of a part of the chromosome carrying the wild-type allele (loss of heterozygosity, LOH). The *APC*-related pathway of transformation is typical of distal (left) colorectal cancers, whereas proximal (right) colorectal cancers more often possess microsatellite instability and defects in mismatch repair genes. A differential pattern of gene expression between the proximal and distal colon may, in part, cause the tissues' susceptibilities to certain pathways of tumorigenesis. Mutations in the *APC* gene lead to the accumulation of hypophosphorylated β catenin protein in the cytosol and later in the nucleus, where β -catenin together with transcription factors (T cell factor/lymphoid enhancer-binding factor) constitutively activate the expression of target genes such as *C-myc* and *cyclin-D1*. Numerous studies have demonstrated that these patients have a higher risk of recurrent adenomas (associated with the size and number of the initially detected adenomas) and / or of developing colorectal cancer than the general population. According to a meta-analysis of 116 studies, the overall prevalence of colorectal cancer in patients with ulcerative colitis is 3.7%. In addition, an estimation of the cumulative colorectal cancer risk according to the duration of ulcerative colitis was calculated to be 2% at 10 years, 8% at 20 years, and 18% at 30 years. The evidence for the link between Crohn's disease and colorectal cancer is less clear than for ulcerative colitis. According to a meta-analysis conducted in 2007, patients with Crohn's disease were found to have a 2.4-fold increase in risk of colorectal cancer, which was however associated with significant heterogeneity (Teppo et al., 1985) (Nishisho et al., 1991) (Powell et al., 1992) (Aaltonen et al., 1993) (Kinzler et al., 1996) (Tetsu O et al., 1999) (Eaden et al., 2001) (Lynch et al., 2003) (Winawer et al., 2003) (Mitchell et al., 2008) (Rapuri et al., 2008) (Xie et al., 2008).

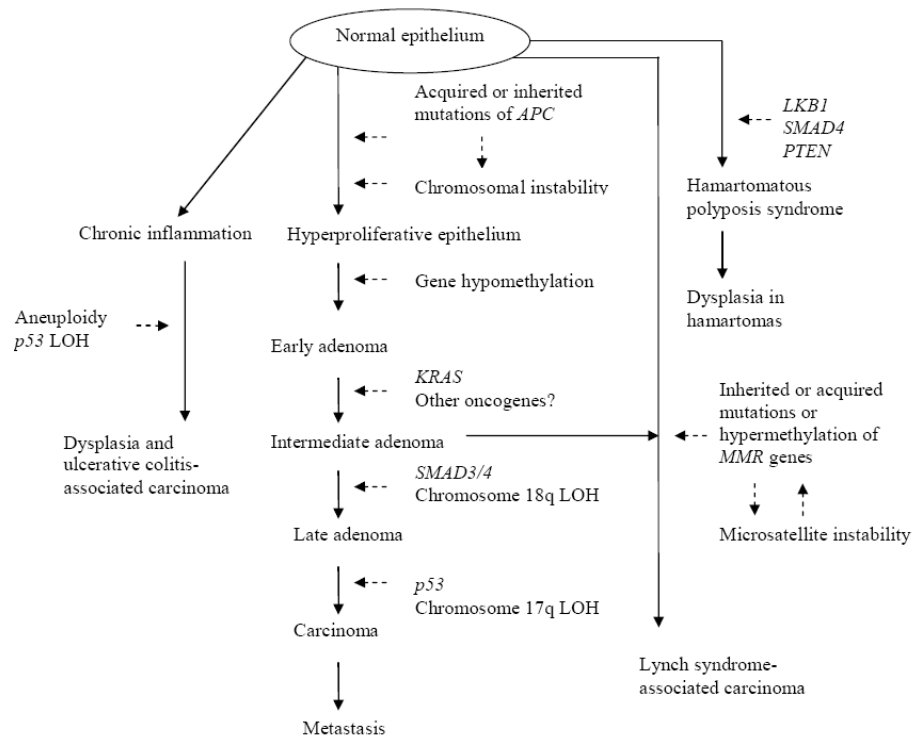


Fig. 8 Colorectal carcinomas arise from a multistage process in which epithelial cells harbour multiple genetic changes in tumour-suppressor genes and protooncogenes. *APC*, adenomatous polyposis coli; *KRAS*, proto-oncogene coding a signalling protein; *LKB1*, serine/threonine kinase gene; LOH, loss of heterozygosity; *MMR* genes, mismatch repair genes; *p53*, a gene coding a multifunction tumour-suppressor protein; *PTEN*, a tumour-suppressor gene coding a protein that attenuates signals originating at tyrosine kinase receptors, e.g. insulin-like growth factor 1 receptor; *SMAD3/4*, genes coding signaling proteins downstream of transforming factor b.

Hormonal factors also seem to play a role in the progression of colorectal cancer in humans since hormone-replacement therapy in postmenopausal women decreases the risk of colorectal cancer. The protective mechanism is unclear, but a decrease in secondary bile acid concentration by oestrogen or a direct effect of oestrogen on epithelial cells has been suggested. A more recent hypothesis involves ER β , which was cloned in 1996 and found also to be present in the intestinal mucosa of the colon. Endogenous oestrogen was also demonstrated to protect against *Apc* induced tumour formation, and this protection was associated with a relative increase in ER β and a decrease in ER α in intestinal tissues. Later the same study group showed that treatment of ovariectomized Min mice with 17- β estradiol (E2) and the phytoestrogen coumestrol, but not the soy isoflavone genistein, resulted in a significant reduction in tumour number. The data reported thus far suggest that both endogenous oestrogen and functional ER β may protect against colon cancer. The proposed actions of lignans in colon tumorigenesis might be direct (inside the

intestinal lumen) or indirect (systemic *via* blood), possibly being mediated through the ER. However, ER-independent mechanisms may also be involved. (Grodstein et al., 1999) (Foley et al., 2000) (Hawk et al., 2004) (Javid et al., 2005).

Environmental factors are likely to cause damage to DNA through direct binding of metabolites (adduct formation) or oxidative stress, whereas repair of such lesions and defence against oxidative stress could be crucial. Single nucleotide polymorphisms result in substantial variation in the capacity of these mechanisms and may be important biomarkers of susceptibility to cancer. Several life style factors and dietary components are suggested to be associated with risk of colorectal cancer. The associations may possibly be due to an increasing level of DNA adducts and oxidative DNA damages. Air pollution is not an established risk factor for development of colorectal cancer in humans, although several studies have shown higher risk among workers exposed to diesel exhaust. DNA adduct levels are increased following occupational exposure among foundry and coke oven workers and among workers exposed to diesel exhaust, while among fire-fighters, traffic exposed policemen and aluminium workers, no associations between occupational exposures and DNA adducts have been found. Exposure to ambient air particles and benzene has consistently been associated with oxidative DNA damages, e.g. high levels of 8-oxoG in lymphocytes (Nielsen et al., 1996) (Goldberg et al., 2001) (Kyrtopoulos et al., 2001) (Sorensen et al., 2003)

According to numerous studies, diet has a very important role in the prevention and causation of colorectal cancer. It has also been thought that the role of diet in colorectal carcinogenesis is particularly important when a poor diet is combined with a generally unhealthy lifestyle, consisting of excess calorie intake and weight gain, physical inactivity and consumption of alcohol. The roles of several foods and nutrients in colorectal carcinogenesis have been investigated. Evidence regarding the positive association between colorectal cancer and intake of red and processed meat is quite consistent. Various review, meta-analysis and observational analytical studies showed a positive association of intake of red meat and processed meat with risks associated with colorectal cancer. For example a meta-analysis of the cohort data showed that every 50g/day increase of red meat intake was associated with a 15% increase in colorectal cancer risk. The elevated risk may be due to an increased endogenous production of N-nitroso compounds (NOC), which may enhance the colonic formation of the DNA adduct O6-carboxymethyl guanine. Cooking meat at

high temperatures, intake of charbroiled or smoked meat lead to the formation of polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines (HCAs) and increased levels of DNA adduct. The levels of some HCAs and PAHs are comparable for red meat, fish and poultry smoked or cooked at high temperatures. Intake of red meat, but not of fish and poultry, increases the luminal contents of N-nitrosocompounds (NOCs) in colon. The increase in endogenous N-nitrosation can be attributed to heme iron, which is 10-fold higher in red meat than in white meat. An increase in the ratio of the consumption of red meat to consumption of fish/chicken was associated with an increase in colorectal polyp risk. Colorectal cancer risk may be negatively associated with fish intake. Intake of fish are reported to be negatively associated with DNA adduct levels, although another study found no effect. The protective effect of fish intake are suggested to be due to the content of n-3 poly-unsaturated fatty acids in fish. High intake of dietary fat has been associated to an increased risk of proximal cancers, while high intake of protein has been associated with an increased incidence of distal cancers. A statistically significant lower risk for colorectal cancer associated with high-fibre intake was showed from the European Prospective Investigation into Cancer and Nutrition (EPIC; 1,721 cases, nine European countries) and in other research. Intake of fruit, vegetables or antioxidant vitamins have been shown to be negatively associated with DNA adduct levels, although some studies found no effect and one study found an effect of increased vitamin intake only in females. The significance of dietary fibres as a protective factor against colorectal cancer remains controversial. However, a large study of European populations (the EPIC study) including 519,978 individuals have confirmed intake of dietary fibres to be protective. To my knowledge no studies has been published concerning intake of fibres and the level of oxidative DNA lesions or DNA adduct formation. Studies in cancer epidemiology and experimental carcinogenesis provided basis for possible mechanisms relating diet and colorectal cancer risk. Investigators initiated animals with carcinogens and then attempted to accelerate or inhibit promotion by modifying the diet. Two possible pathways to explain these puzzling results are explained as shown in Figure 9 in a local irritation. The irritation produces a focal inflammatory response that activates COX-2 (cyclooxygenase) and generates prostaglandins from arachidonic acid. This activates inflammatory cells which generate reactive oxygen intermediates (ROIs) that are mutagenic and mitogenic and promote carcinogenesis.

In the second pathway, a defect in the epithelial barrier results in an electrolyte imbalance, an efflux of potassium and an influx of sodium and calcium in epithelial cells. The electrolyte disturbance results in an oxidative stress and ROI generation as the epithelial crypt cells cope with restoring intracellular electrolytes to their normal values.

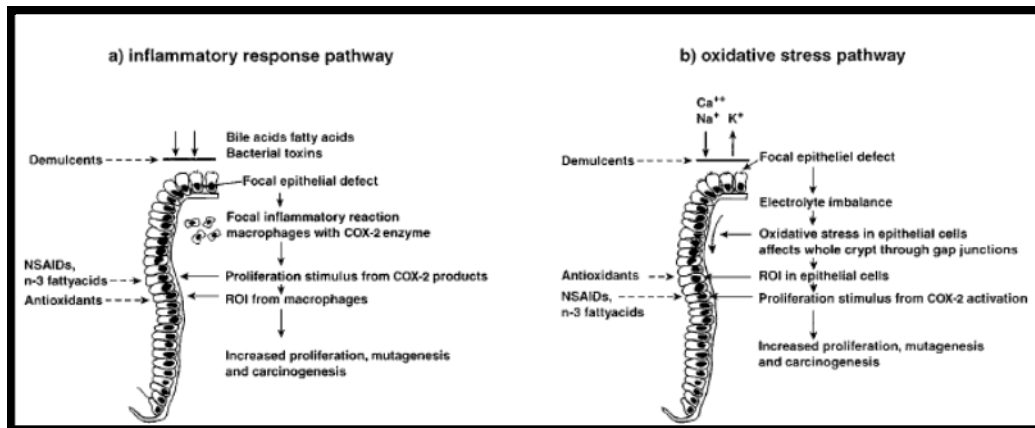


Fig. 9 Two pathways involved in the focal epithelial defect mechanism inferred from the inhibition of carcinogenesis by demulcents and antioxidants.

The resulting focal proliferation and mutagenesis give rise to aberrant crypt foci and adenomas. The process is inhibited by: (a) demulcents confined to the colonic lumen that “repair” the surface; (b) anti-inflammatory agents; or (c) anti-oxidants. Fruits and vegetables contain dietary fiber and phytochemicals that can act as antioxidants and inhibit colon carcinogenesis. There is limited evidence for a preventive effect of intake of fruit and vegetables for cancer in colon and rectum. Findings from case-controls studies suggest that there is a positive and dose dependent association between dietary energy intake, body mass index (BMI) and physical activity and colorectal cancer risk (McMichael et al., 1985) (Rothman et al., 1990) (Gomaa et al., 1993) (Rothman et al., 1993) (Skog et al., 1995) (Bingham et al., 1996) (Bruemmer et al., 1996) (Bruce et al., 2000) (Yoon et al., 2000) (Bingham et al., 2003) (IARC, 2003) (Larsson et al., 2003) (Pierre et al., 2003) (Norat et al., 2005) (Sinha et al., 2005) (Lewin et al., 2006) (Cross et al., 2007) (Johnson, 2007).

2.1 Lignans and colorectal cancer studies

During the last two decades, the health effects of lignans in humans have received much attention. Many studies have established that there exists an inverse relationship between lignans consumption and reduced cancer risks. Later, the incidence of so-called Western diseases, including colon cancer, was proposed to be explained by decreased intake of lignans and isoflavones. In colon cancer epidemiological studies, the relationship between lignans intake and colon cancer is summarized by a publication that demonstrates that a substantial reduction of colorectal adenoma risk is associated with a high plasma level of lignans metabolites. In an American based colon cancer population case control study involving 1095 cases and 1890 control subjects, the authors suggest that dietary lignans intake is associated with a significant reduction in colorectal cancer risk. They did not observe any evaluated interactions between polymorphic genes that encode enzymes possibly involved in metabolism of phytoestrogens like catechol O-methyl transferase, GSTs and others and the risk of colon cancer. No association was found between plasma enterolactone level and risk of colorectal cancer or risk of cancers of the colon and rectum separately in one unpublished study. Many studies involving cancer inducible animal models and cell culture models test the potential benefits of lignans as anti-cancer agents by using either purified lignans preparations or foods rich in lignans. Foods rich in plant lignans, when tested in animal experimental and *in vitro* studies, also have effects that may protect against cancers of the gastro-intestinal tract. Carcinogenesis or tumor markers in animal models is induced with either azoxymethane or N-nitroso N-methylurea (NMU) or 7, 12 dimethylbenz (α) anthracene (DBMA) treated along with lignans or lignans enriched diets. In one case, spontaneous carcinogenesis animal model, Apcmin (mutated adenomatous polyposis coli gene) were used. In two cases, human breast cancer cell lines or melanoma cancer cell lines xenografted into the animal model caused cancers. In almost all animal models, lignans supplementation/treatment is started before induction of the carcinogenesis except in a few cases, where it was after carcinomas had been established. Lignans or SDG equivalent diets was the most common lignans diet used except two studies that used ENL injected intravenously. Lignans plasma amounts are directly correlated with reduced cancer risk as shown in some population studies and proved in animal and cell culture models. Unlike epidemiological studies, more

consistent results are evident in experimental animal and cell culture studies. The impact of lignans on colon carcinogenesis has been studied mainly with carcinogen-treated rats, with formation of early precancerous lesion (aberrant crypt foci, ACF) or tumour in the colon being used as an end point. The results indicate that lignans are chemo-preventative against initial stages of carcinogenesis as seen by reduction in number of early carcinogenesis related markers, such as number of ACF (aberrant crypt foci), size and number of adenoma, size and number of tumors and number of metastases. Flaxseeds decreased the number of aberrant crypts (AC) and ACF in the distal colon of carcinogen-treated rats by 41-53%. In another experiments carcinogen-treated rats were fed either a control diet or a diet supplemented with flaxseeds (at level of 2.5% or 5%) or pure SDG, the number of AC per focus (AC multiplicity) was significantly reduced in the distal colon of flaxseed and SDG groups. The protective effect was partly due to SDG, and there was a negative association between total urinary lignans excretion and AC multiplicity. Another study reported that multiple intestinal neoplasia (Min) mice fed rye bran had the lowest adenoma number and incidence of colon adenomas (33%) of all experimental groups. In addition flaxseed consumption did not explain adenoma formation in Min mice. Only one study has evaluated the role of pure lignan isolate in colon carcinogenesis. When pure lignan arctiin from *Arctium lappa* (burdock) seeds were fed to carcinogen-treated rats, the number of ACF per colon was significantly decreased in the group fed 0.02% arctiin during the initiation period, and in groups fed either 0.02% or 0.2% arctiin during the post-initiation period. Two studies have reported the effects of flaxseeds and pure SDG on experimental metastasis of melanoma cells in mice. In both studies, B16BL6 murine melanoma cells were injected into C57BL/6 mice, which resulted in the formation of metastatic lung tumours. Flaxseeds at levels of 5% and 10% reduced tumour area, tumour volume and the median number of tumours by 54% and 63%, respectively. In the second study, SDG at 147 and 293 mmol/kg reduced tumour area and tumour volume. However, only the highest SDG level (equivalent to a 10% flaxseed diet) reduced the median number of tumours by 53%. This indicates that flaxseed *per se* is more potent in preventing metastasis than an equivalent amount of pure SDG. SDG from flaxseed, arctiin, 7-hydroxymatairesinol and ENL have been the most potent anticarcinogenic compounds in animal studies. Unlike the animal model experiments, the cell culture studies on human colon, prostate and breast cancer cell

lines mostly use ENL and END. *In vitro* studies suggest that ENL is capable of inducing quinone reductase, a phase II detoxification enzyme in Colo205 cells. A study suggested ENL inhibited a human colon cancer cell line, SW480 in a time and dose dependent manner. The effect may be additive when both ENL and END are combined. ENL is also shown to induce apoptosis and inhibit growth in human colon cancer cell line, colo-201. An intervention with linseed containing bread reduced fecal water-induced genotoxicity and subsequently DNA damage in HT29 cells. In four other human colon tumor cell lines; LS174T, CaCo-2, HCT-15 and T-84, ENL and END at 100 μ M concentration reduced cell proliferation. In a study, enterolactone (IC 50 of 57 μ M) is more potent than END (IC 50 of 100 μ M) to reduce cells growth. Therefore growth inhibitory effects in the cancer cell lines can be explained by several mechanisms such as anti oxidant, estrogenic and anti-estrogenic mechanisms (the potential antitumorigenic effects of lignans or lignans-rich foods may involve both ER-mediated and ER-independent mechanisms) among others (Adlercreutz et al., 1980) (Maria et al., 1992) (Mousavi et al., 1992) (Serraino et al., 1992) (Thompson et al., 1997) (Sung et al., 1998) (Thompson et al., 1998) (Li et al., 1999) (Hirose et al., 2000) (Lin et al., 2001) (Lin et al., 2002) (Saarinen et al., 2002a) (Cos et al., 2003) (Danbara et al., 2005) (Qu et al., 2005) (Kuijsten et al., 2006) (Pajara et al., 2006) (Thompson et al., 2006).

§ 3. BUCKWHEAT AND BIOACTIVE COMPOUNDS

The name “buckwheat” comes from the Anglo-Saxon words *boc* (beech) and *whoet* (wheat) because the seed resembles a small beech nut. Buckwheat is neither a nut nor a cereal like wheat, but rather a pseudocereal whose history dates back over 1000 years. Cereals at their most basic structure are “one-seeded” fruits containing a small embryonic germ and a larger, starchy endosperm surrounded by an outer aleurone layer and a hull. Like cereals, the seed of the buckwheat plant contains a germ, endosperm, aleurone layer, and a hull. However, buckwheat is not a part of the cereal or grain family (Gramineae) but comes from the family of Polygonaceae which is generally referred to as the buckwheat, rhubarb or sorrel family. Buckwheat can grow to be anywhere from 60 and 120 cm, produces white or pink blossoms with five petals. Buckwheat is a broad-leaved herbaceous and can be divided into groups of species: annual and multiannual. Although it contains the same tissue components as cereals, buckwheat has different tissue features. Buckwheat is a dicotyledon while grains like wheat and corn are monocots. Dicotyledons contain two cotyledons or “seed leaves” which store and absorb food for the plant during germination and primary growth. The foliage of dicotyledons contains netlike vascularization and the vascular structures are organized in a ring like structure in the stem. The buckwheat grain consists of a triangular seed (covered by a hull pericarp) with two cotyledons running through the endosperm and surrounding it. The exact shape, size, and color of the seed may vary depending on the species and variety. The hull may be a glossy or dull brown, black or gray. This pseudocereal is uniquely rich in proteins 8-16%, contain 60-75% carbohydrate, and varying levels of lipid, although most contain between 2-3%. In a study dehulled buckwheat groats were found to contain 75% starch the major carbohydrate in buckwheat, 13.9% protein (these have a high biological value, but relatively low true digestibility) and 2.3% lipid. There is a prevalence of unsaturated fatty acids C18:1, C18:2, C18:3 and C20:1 and highest content is in the embryo (9.6-19.7%), the endosperm contains 2 - 3% and the hulls 0.4 - 0.7 %. The ash content of buckwheat varies from 2 - 2.2 %, depending upon the variety. An estimate of the whole groat is that groat starch contained 55% starch, 12% protein, and 4% lipid (Fig.10). Most of the protein and lipid were found in the bran and embryo tissue. The main protein fraction is globulin, which represents almost half of all proteins. A characteristic feature of buckwheat proteins is a very

low content of prolamins. Unlike wheat and other cereals, buckwheat does not contain gluten, a protein used in building volume in breads; however, this may be advantageous for people with celiac disease. Common (*Fagopyrum esculentum* Moench., originates from Southwest China) and Tartary buckwheat (*Fagopyrum tataricum* Gaertn.) are the only two of the many buckwheat species known that are cultivated for human consumption. The amino acid profile of buckwheat was found to be different from grains and similar to that of other dicotyledons such as soybeans with higher amounts of lysine (5-7%, is deficient in major cereal crops), methionine, cystine, arginine, and aspartic acid. In common buckwheat bran, protein content was 21.6%, and in tartary buckwheat, 25.3%. Buckwheat groats contained about 7.0 g/100 g DW total dietary fiber; of which 2.2 g/100 g DW was insoluble and 4.8 g/100 g DW was soluble.

Crop	Protein	Ash	Lipids	Dietary fibres		
				Soluble	Insoluble	Total
Wheat	11.5	1.7	1.0	1.0	1.5	2.4
Common buckwheat	11.0	2.6	3.4	1.2	5.3	6.5
Tartary buckwheat	10.3	1.8	2.5	0.5	5.8	6.3
Oats	12.6	1.8	7.1	3.3	4.9	10.2
Rye	11.7	1.5	1.8	3.6	10.0	13.6
Spelt wheat	13.5	1.9	2.5	0.6	5.4	6.0

Fig. 10 Chemical composition of some cereals flours (% dry matter basis).

It also contains an abundance of minerals (zinc, manganese, selenium, iron, phosphorus, and copper) and vitamins (B1 and B2, total B vitamin content is higher in tartary buckwheat than in common buckwheat). Common buckwheat is the most widely consumed buckwheat species with its advantages of sweet taste and large, easily dehulled seed. Researchers are very interested in using tartary buckwheat because its content of bio-active compounds is much higher than in common buckwheat, which has been traditionally utilized throughout the world. Tartary buckwheat has the disadvantages of bitter taste, small seed size, and a hull that firmly adheres to the testa thus making de hulling extremely difficult. As with grains, in order for buckwheat to be used as a food product, it must first be milled. In the most basic milling process, the outer hull is removed from the seed to produce a groat. The

hulls of the buckwheat can be sold for special pillows. The dehulled seed, or groat, is used in breakfast cereals and milled into grits (coarsely ground groats are called grits). The groat can then be ground further into several fractions with varying levels of the aleurone layer remaining and can be used for porridges or in breads. Roasted groats are called kasha, and are sold in whole and granulated forms. Both kasha and groats can be baked, steamed or boiled for nutritious alternatives to potatoes and rice. Roasted groats (kasha) are used in Eastern European ethnic dishes. In Eastern Europe, buckwheat flour made from the aleurone layer of the groats is called Farinetta™ and is used in cooking similar to wheat flour to make in breads, bakery products, pancakes, alone in some dishes, such as polenta and zganci. Buckwheat flour in bread mixes comprises only 30% to 40% of the total. It may also be used in desserts, ice cream, dietetic foods, pancake mixes, canned meat products, canned vegetable products and dried breakfast cereals. Pasta produced from a mixture of wheat and buckwheat flour has been characterized to possess shorter cooking time. Buckwheat can be used to produce extruded cereal and snack products. Extruded buckwheat products are of very high nutritional quality when compared with products extruded from wheat. Flour made from the entire buckwheat groat (Supreme flour) can be used in breads, bakery products, extruded snacks, pancakes, and pasta. Fancy flour made from the whiter endosperm portion contains high amounts of starch and can be used in food products like soba noodles a Japanese staple or Makguksu, Naengmyeon and Mug which are kinds of Korea traditional noodles and curds, respectively. The specific character of the proteins allows buckwheat to potentially be used in extruded products targeted to special nutritional needs. In addition to being used as a direct food source, buckwheat blossoms also provide nectar for honey bees. Buckwheat is ideal in that its blooms last up to a month later in the year than other honey-producing crops, providing a later harvest for beekeepers. The honey from buckwheat nectar tends to be darker and taste stronger than other honeys. Other products made from buckwheat are green buckwheat tea, buckwheat beer and vinegar, spirit, buckwheat sprouts and fresh green plant parts used as a vegetable. Buckwheat can also be used as a feed source for livestock and wildlife. Aside from its food potential, buckwheat crops are also useful for ground maintenance and to eradicate weeds (potent against sowthistle, Canada thistle, quackgrass, creeping Jenny, Russian knapweed, leafy spurge, and perennial peppergrass) and also helps to revitalize soil by aerating the soil with its

shallow and fibrous root system. Buckwheat takes little time to grow (10-12 weeks) which makes it ideal as an emergency crop for crops that fail. Buckwheat is an ancient plant whose origins lie in China where it was believed to have been first cultivated around 1000 AD. About 500 years later it was introduced in Europe and brought over to the Americas during the early colonial period. Today buckwheat is grown in several areas throughout the world including India, Tibet, Bhutan, China, Japan, Russia, Australia, Canada, the United States, Germany, Poland, Slovenia, Italy, and the Ukraine with Russia being the highest producer followed by China. In Italy, buckwheat is grown in the Alpine region (Valtellina and Val Venosta) and used for preparing typical, regional food products like “Pizzoccheri”. Despite its past history as a food, feed, and ground enhancement product, buckwheat production has seen a decline within the United States over the last 100 years. Several factors account for the decline of production in buckwheat in the United States due to the lack of financial support and the variability in production. Production varies unpredictably from cultivar to cultivar and from plant to plant. Even though the plants blossom profusely, only 10-20% produce seeds. Buckwheat plants may produce anywhere from 10 to over 200 seeds. Buckwheat seeds also does not ripen evenly. This creates a variety of yields from only 200 kg/ha to over 3,000 kg/ha. Research into breeding more reliable varieties has been slow in the western hemisphere, although newer breeds from Canadian programs have shown improvement over older varieties and Russian and Chinese production have benefited from research efforts. In addition to financial support and production problems, domestic markets for buckwheat products have declined over the years. In effect, after one year of storage buckwheat is considered to be of inferior quality. Products made from buckwheat tend to be darker in color and have a more “full-bodied taste” which some consumers find disagreeable. Buckwheat may also elicit some allergic reactions in both humans and animals if consumed in large quantities. Allergic reactions are caused by ingestion of allergenic buckwheat proteins. Despite its domestic decline as a staple food and feed source, recent research into the nutraceutical aspects of buckwheat is providing a new perspective for future buckwheat products. Buckwheat is a natural functional food, because it positively affects the human organism biologically (for example low glycemic index, reduced capillary fragility, bloody pressure and have anti-inflammatory, antioxidant, antiviral activities, improving the constipation and obesity conditions) without the necessity of

adding any other components. It has been claimed that buckwheat protein is the active ingredient responsible for cholesterol-lowering activity. In rats fed a diet containing 20% buckwheat protein, there was a 32% reduction in serum cholesterol compared with the same amount of casein. Both buckwheat flour and buckwheat protein were equally effective in reducing plasma TC by 32%. It has been shown that buckwheat protein is more effective than soybean protein in lowering plasma cholesterol in hamsters fed a high cholesterol diet. Buckwheat sprout has been also found to reduce plasma triacylglycerol (TC), TC/HDL-C (high-density lipoprotein cholesterol), and LDL-C (low-density lipoprotein cholesterol /HDL-C in hamsters. Now, with greater concern for human health and for functional food, for sustainable agriculture and for ecological growing a great interest for these plants has appeared. Buckwheat has been found to contain several natural components that make it advantageous for use with diabetes and cardiovascular disease patients. One component that buckwheat groats have been found to contain are phytochemicals which may have antioxidant properties. Studies found that whole buckwheat contained six known non-nutritional substances such flavonoids (rutin, orientin, vitexin, quercetin, isovitexin, and isoorientin) with most being concentrated in the hull and only rutin and isovitexin being found in dehulled buckwheat seeds. Their content depends on many factors such as: climatic and agrotechnical conditions in cultivation and harvesting, ripeness of the material, harvest time, storage conditions, effect of genetic factors and varieties-dependent variability. Rutin is the most dominant medicinal compound in buckwheat and is a substance that has pharmacological effects. In a study of Canadian buckwheat was found that phytochemicals such polyphenols content varied with cultivar and environment and that buckwheat also contained components other than flavonoids which gave it antioxidant properties. Tartary buckwheat is known to have a high content of rutin and other polyphenols, and thus also higher than common buckwheat. The content of rutin in tartary buckwheat is determined as up to 3% dry weight (DW) in the herb, while quercitrin values were in the range of 0.01% to 0.05% DW. Only traces of quercetin were detected, in just some of the samples. Tartary buckwheat seeds contained more rutin (about 0.8 to 1.7% DW) than common buckwheat seeds (0.01% DW). Rutin, quercetin and other polyphenols content in seeds depends on variety and growing conditions. For this reason is important to analyze every sample of pseudocereal before to do experiments. Tartary buckwheat seeds contained traces of

quercitrin and quercetin, which were not found in common buckwheat seeds. Although flaxseed is the richest source of plant lignans (301 mg/100 g), containing 75–800 times more than other oilseeds, cereals, legumes, fruits and vegetables, buckwheat also contains a considerable amount of these compounds. The total lignan content of cereals species can be in the following order: rye > wheat > oat > spelt > wheat > Japanese rice > wild rice > buckwheat > barley > amaranth > corn > millet > red rice > brown rice ranged for grain products from 7 to 764 µg/100 g. The amount of concentrations of all lignans in bran buckwheat is around 1500 µg/100 g as reports Smeets et al. 2007. In animals, the excretion of END and ENL is measured in the urine when different plant components were included in the diet. Results show that buckwheat provided the third highest amount of excreted lignans among many cereals. Another group of phytochemicals associated with buckwheat are fagopyritols or “galactosyl derivatives of D-chiro-inositol” which have potential use for glycemic control in type II diabetics. Buckwheat proteins are also been found to be beneficial. In effect a study shows that rats fed whole buckwheat protein products had lower plasma cholesterol levels than rats fed casein. These results were attributed to higher neutral sterol excretion and lower buckwheat digestibility compared to casein. Comparative studies show the effect that buckwheat protein, casein, and soy protein had on gall bladder excretions and plasma cholesterol in hamsters. They found that consumption of buckwheat proteins elicited higher sterol secretion, lower plasma and liver cholesterol levels, and fewer instances of gallstones than soy protein or casein. Increases in buckwheat usage as a food source because it not only provides nutrition but also nutraceutical advantages may result in an increase in its production in the western hemisphere as well as throughout the world. A particular attention regard the use of this pseudocereal as sprout. Biochemical composition and utilization of buckwheat sprouts as a valuable, functional vegetable has been reported. Buckwheat sprouts have light-yellow cotyledon and bright-white hypocotyl, so their appearances are very similar to those of soybean sprouts. Based on the data of the literature it can be stated that the original composition of the seeds essentially changes during germination. The buckwheat sprouts are outstanding sources of protein, vitamins and minerals and they contain such in the respect of health-maintaining important nutrients. The free sugars in both buckwheat seeds and early stages of seedling were mainly sucrose and maltose, but its composition was gradually changed as seedling days increased and glucose and fructose were detected

as the main free sugars of buckwheat sprouts. Lysine contents of buckwheat sprouts were notably higher amount than buckwheat seeds and the other cereals. Rutin one of a group of flavonoids is abundant in buckwheat sprouts and its contents were increased about 27 times than buckwheat seeds. Buckwheat sprouts have a significantly higher antioxidant activity than seeds, result of difference in the content of total polyphenols and other important bioactive compound. Buckwheat sprouts have very soft and mild flavor and slightly crisp texture, furthermore buckwheat sprouts dose not have beany flavour as in soybean sprouts but have a very attractive fragrance. For these characteristics, buckwheat sprouts could be used as fresh vegetable, salad and various purpose including natural vegetable juice material. Freezed dried buckwheat sprouts is powdered and mixed to make rice cake, bread, and snack. Dried powder of buckwheat sprouts is also mixed in media to cultivate artificially edible and medicinal mushroom. As this sprouts are consumed at the beginning of the growing phase, their nutrient concentration remains very high and studies show that maximum yields of most nutrient occurred on day 8 sprouting. Kim et al. (2007) sprouted buckwheat for a period of 1-10 days in a glass house under low light conditions and determined the chlorogen acid and flavonoid content including the C-glucoside flavons (orientin, isoorientin, vitexin, isovitexin) as well as rutin and quercetin. Rutin content of one meal portion (on average 20-30 mg/g) was 30 times higher than in the root and pericarp. By analyzing the radical capturing capacity of the sprouts by the 2,2-diphenyl-1-picril-hydrazil method it was established that it increased significantly for six to ten days in the portion from 1.52 to 2.33 μmol for the one buckwheat variety whereas for the other it increased from 1.46 to 2.09 μmol but the difference between the two sorts of buckwheat was not significant. On the basis of their investigations they recommend the consumption of the buckwheat sprouts during the everyday meals. However actually little is known about buckwheat sprouts and powder of these (at present it is not used as functional ingredient in the formulation of foodstuffs) and no study characterized the phytoestrogens lignans in buckwheat sprouts (or in dried powder of sprout) (Eggum et al., 1980) (Pomeranz et al., 1983) (Thompson et al., 1991) (Hoseney et al., 1994) (Kitabayashi et al., 1995) (Edwardson et al., 1996) (Oomah et al., 1996) (Kayashita et al., 1997) (Zheng et al., 1998) (Dietrych-Szostak et al., 1999) (Rickard et al., 2000) (Starr et al., 2000) (Steadman et al., 2000) (Tomotake et al., 2000) (Li et al., 2001) (Steadman et al., 2001) (Sun Lim Kim et al., 2001) (Vavreinová et al., 2001)

(Vinning et al, 2001) (Wieslander et al., 2001) (Bonafaccia et al., 2003) (Fabjan et al., 2003) (Stibilj et al., 2004) (Kim et al., 2007) (Tomotake et al., 2007) (Chen et al., 2008) (Ishii et al., 2008) (Lin et al., 2008).

§ 4. AIMS TO THE STUDY

The interest in the potential health effects of dietary phytoestrogens has increased the epidemiological and clinical studies related to effects of this bioactive compounds on bone density, cardiovascular health, cancer prevention, cognitive ability and menopausal symptoms. However the prevalence of phytoestrogens and lignans in our diets and the biological effects that they may cause need to be fully examined. Development of colon cancer, a common form of cancer in Western countries, is related to diet and dietary bioactive compounds. In recent times, especially in all industrially developed countries, consumers are becoming more interested in foods which offer an added value in terms of health benefits. Epidemiological studies indicate a protective effect on various degenerative disease of buckwheat consumption. Because this properties buckwheat seeds and sprouts has been and will be used as an important raw material for functional food production rich in bioactive compounds. In accordance with the above considerations, in this PhD thesis, I evaluated some of the biological effects of phytoestrogens, in particular, of lignans. I selected and characterized buckwheat seeds and sprouts as potential rich food sources of polyphenol and pytoestrngens. In particular I evaluated the effects of different lignans *in vitro* on human colonic cancer cell lines HT29 and HCT8. The first aim was to evaluate the effects of lignans on cellular proliferation. The second objectives was to analyze the mechanisms trough which lignans are involved in modulation of cell cycle and on the expression of the protein related in the regulation of cell cycle on the human colonic cancer cells. I also investigated the effect on the redox state of metabolites of lignans in cultured human colonic cancer cells. In addition to setting up a preliminary basis proof-project for formulation of functional food containing significant levels of biologically active compounds, I developed a cultural procedure for buckwheat sprouts after selection of cultivar of seeds. The objective of these last experiments was not only to examine cultured buckwheat sprouts and to evaluate the yields of harvest, but also to analyze the amount of total phenolic contents, both in seeds and in sprouts. The research was conducted on the only two of the many buckwheat species known that are cultivated for human consumption, Common and Tartary also known respectively as *Fagopyrum esculentum* Moench. and *Fagopyrum tataricum* Gaertn.

§ 5. MATERIALS E METHODS

Cell Lines, treatments and cell growth assay

HT29 and HCT8 human colon carcinoma cell lines with colorectal and ileo-cecal oringin respectively, were obtained from American Type Culture Collection (ATCC). The medium for both cells lines were supplemented with 2 mmol/L of L-glutamine and 10% FCS (foetal bovine serum), 100 IU/ml penicillin and 10mg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. For the experiments, cells were seeded onto plates (96 microwell, 2x10⁵ cells/well) cells and allowed to adhere for 24h until the 60-70% confluence. Lignans (Sigma Chemical and ChromaDex), SECO (secoisolariciresinol), MAT (matairesinol), END (enterodiol), ENL (enterolactone), SDG (secoisolariciresinol diglucoside) was dissolved in DMSO (250 mmol/L) and stored at -20°C. HT29 in Dulbecco medium and HCT8 were maintained in RPMI 1640 (Gibco, Carlsbad, CA). The final DMSO concentration in all cultures was 0.2%, a concentration that did not alter cell growth or cell cycle measurements compared with the vehicle-free media. Working solutions of lignans were then made by serial dilutions of the stock solutions with cell culture medium. Then the medium was replaced with fresh medium supplemented with 150 µmol/L of lignans. At 24 h and 48 h the cells were detached with trypsin-EDTA. The effects of this compounds on cell proliferation were determined by the BrdU (5-bromo-2'-deoxyuridine) cell proliferation kit (Roche). The BrdU (10 µg/ml) was added to cells cultured in microplates, then the cells are incubated. The technique is based on the incorporation of the pyrimidine analogue BrdU (instead of thymidine) into the DNA of proliferating cells. After its incorporation into DNA, BrdU is detected by an immunoassay. Bound anti-BrdU-POD (peroxidase labelled anti BrdU) is detected by a substrate reaction, then quantified by a microplate reader (Tecan Infinite F200, Germany). All experiments were performed in triplicate.

Cytotoxicity assay

The viable and nonviable cells were detached with trypsin-EDTA, washed with cold PBS (Phosphate Buffer Saline) and were discriminated by tripan blue dye exclusion. The viable cell numbers in treated cells were compared with that in vehicle controls.

Cell cycle analysis

Cell cycle was analyzed by iodure propidium (PI 50 µg/ml, 0.1% sodium citrate e 0.1 % di Triton X-100). The floating and trypsinized adherent cells were collected and washed with PBS. The cell pellets were resuspended in 300 µL of PBS and finally the cells were stained with 0.5 µl/ml of PI solution for 30 min and incubated at 4°C. Fluorescence intensity was analyzed by a FACScalibur flow cytometer (Becton-Dickinson) was performed with an excitation at 488 nm and an emission at 630 nm. CellQUEST software was used for data acquisition (5x10³ cells events/sample).

Western blot analysis

Triton lysis buffer was used to extract protein from treated cells. Protein concentration was measured by spectrophotometer (BioRad Protein Assay Dye Reagent Concentrate) by Bradford method. 40 µg of whole cell proteins was electrophoresed on 12% SDS polyacrylamide gels (SDS-loading buffer: glycerol 10%, β-mercaptoethanol 10%, SDS 2%, Tris-HCl pH 6,8 625mM e blue of bromophenol 0,1%) and transferred to pure nitrocellulose membrane by elettroblotting. Costant ampere 25 mA, for 90-120 minutes in Running-Buffer 1X content Tris, Glicin e SDS (Bio-Rad Laboratories). The membrane Hybond c Extra (Amersham, NY, USA) was blocked in 5% non-fat dry milk overnight at 4°C and washed with 0.5% PBS-tween 6 times for 5 min each time. The membrane was incubated with rabbit polyclonal IgG against cyclin D1 and then with another specific IgG P21 (Santa Cruz Biotechnology) for 1 h at room temperature. The control used was GADPH. After washing, the membrane was incubated in anti-rabbit IgG of cyclin D1, P21 and GDPH for 1 h at room temperature. Bands were detected by a photograph plate. Band intensities were quantified and analyzed with the analyst software program ImageJ.

Analysis of intracellular generation of ROS (reactive oxygen species)

The generation of ROS was measured using dichlorofluorescein fluorescence. Treated cells were washed once in PBS, incubated with 20 µM of H₂DCF-DA (2'-7'-dichlorofluorescein diacetate) for 30 min at 37°C followed by immediate analysis in the flow cytometer. Analyses were performed on 1x10⁴ cells in FL1 (530 nm).

Source of Buckwheat and cultivation of Buckwheat Sprouts

Common buckwheat seeds (*Fagopyrium esculentum* Möench) and tartary buckwheat seeds (*Fagopyrum tataricum* (L.) Gaertn.) were supplied by National Institute of Research on Food and Nutrition of Rome, Italy. Buckwheat sprouts were cultured by production system which was constructed to control the temperature (18°-20°) and water spray at a regular interval for eight days. Seeds were soaked in water for 6 hour at room temperature and then put into a germination bag. The culture vessels were specially designed to remove the pericarps from buckwheat sprouts. The sprouts 8 day after seeding were harvest (a length mean 5-6 cm), immediately dried in a oven (Temperature 45-50° C for 48 hours) and then finely grounded and pulverized to be used for preparing the samples of chemical analysis.

Total phenolic contents (TPC): Buckwheat preparation experimental design

Grounded dry seeds was weighed accurately (1 gr) into a test tube and extracted at room temperature with 80% aqueous methanol under agitation using a magnetic stirrer for 2 hours. The mixtures were centrifuged at 12000 g for 10 min at 4 ° C and the supernatants were collected. The TPC of extracts was determined (according to the method of Shahidi and Naczki, 1995) using the Folin–Ciocalteu reagent and are calculated from a standard curve using a linear range of concentration of ferulic acid. Extract (0,25 ml) was added to 0,25 ml of freshly Folin–Ciocalteu reagent (after water dilution 1:2 v/v) into test cuvettes. 0,5 ml of sodium carbonate solution (75 g/l) and 4 ml of water were added to the mixture. The absorbance of all samples (resulting blue color) was measured at 725 nm using the spectrophotometer UV-Vis (Uvicon 942, Kontron) after incubating at room temperature for 25 minutes and centrifuged at 2100g for 10 minutes. Results have been expressed as mg/g of dry matter. The same analytical procedure was used to determine TPC of dried powder of buckwheat sprouts.

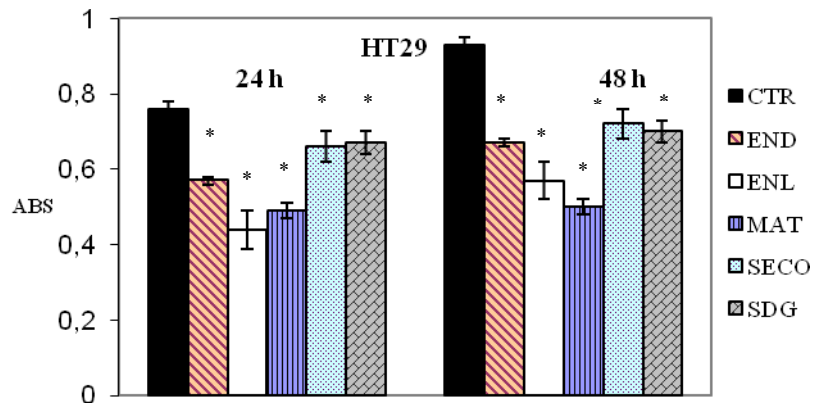
Statistical analysis

All values were given as mean $\pm$ SD for at least triplicate experiment. Differences between two groups were analyzed by unpaired Student's t-test. Data were analyzed by the ANOVA/T Test. Values of $P < 0.05$ were considered significant.

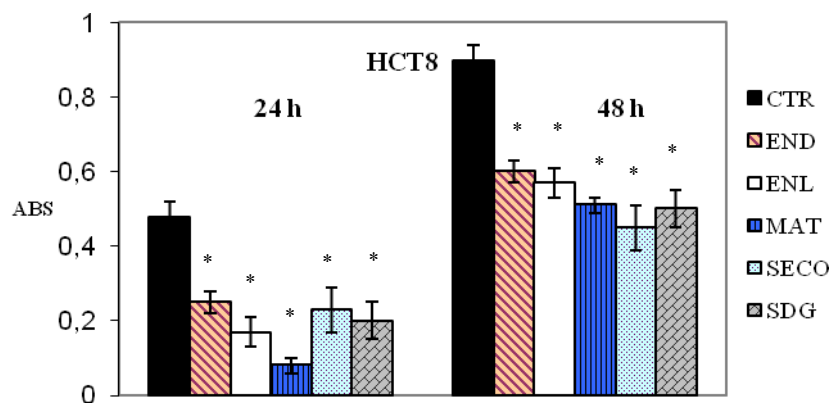
§ 6. RESULTS

6.1 Cell growth

Human cancer cells HT29 and HCT8 treated with lignans showed a growth inhibitory effect. The effect of treatment with lignans SECO (secoisolariciresinol), MAT (matairesinol), END (enterodiol), ENL (enterolactone), SDG (secoisolariciresinol diglucoside) at 150 $\mu\text{mol/L}$ for 24 and 48h on the growth of HT29 and HCT8 cells is reported in Figure 11. By the cell proliferation kit using BrdU (5-bromo-2'-deoxyuridine) it can be observed that treatment decreases in cell numbers compared with the control. The inhibition increased in HCT8 cell because they are in a different state of differentiation compared with HT29. All experiments were performed in triplicate.



(A)



(B)

Fig. 11 Effect of 150 µmol/L lignans SECO (secoisolariciresinol), MAT (matairesinol), END (enterodiol), ENL (enterolactone), SDG (secoisolariciresinol diglucoside) on the growth of HT29 (A) and HCT8 (B) cells. Values are means $\pm$ SD of 4 different experiments performed in triplicate, results are significant $P \leq 0.05$ (*).

6.2 Cytotoxicity

The viable and nonviable cells were discriminated by tripan blue dye exclusion. The viable cell numbers in treated cells (24 and 48 h) were compared with that in vehicle controls. Cell viability was generally >80% in adherent cells, and the treated cells did not differ from the vehicle controls (Fig. 12). The final DMSO concentration in all cultures was 0.2%, a concentration that did not alter cell growth or cell cycle measurements compared with the vehicle-free media.

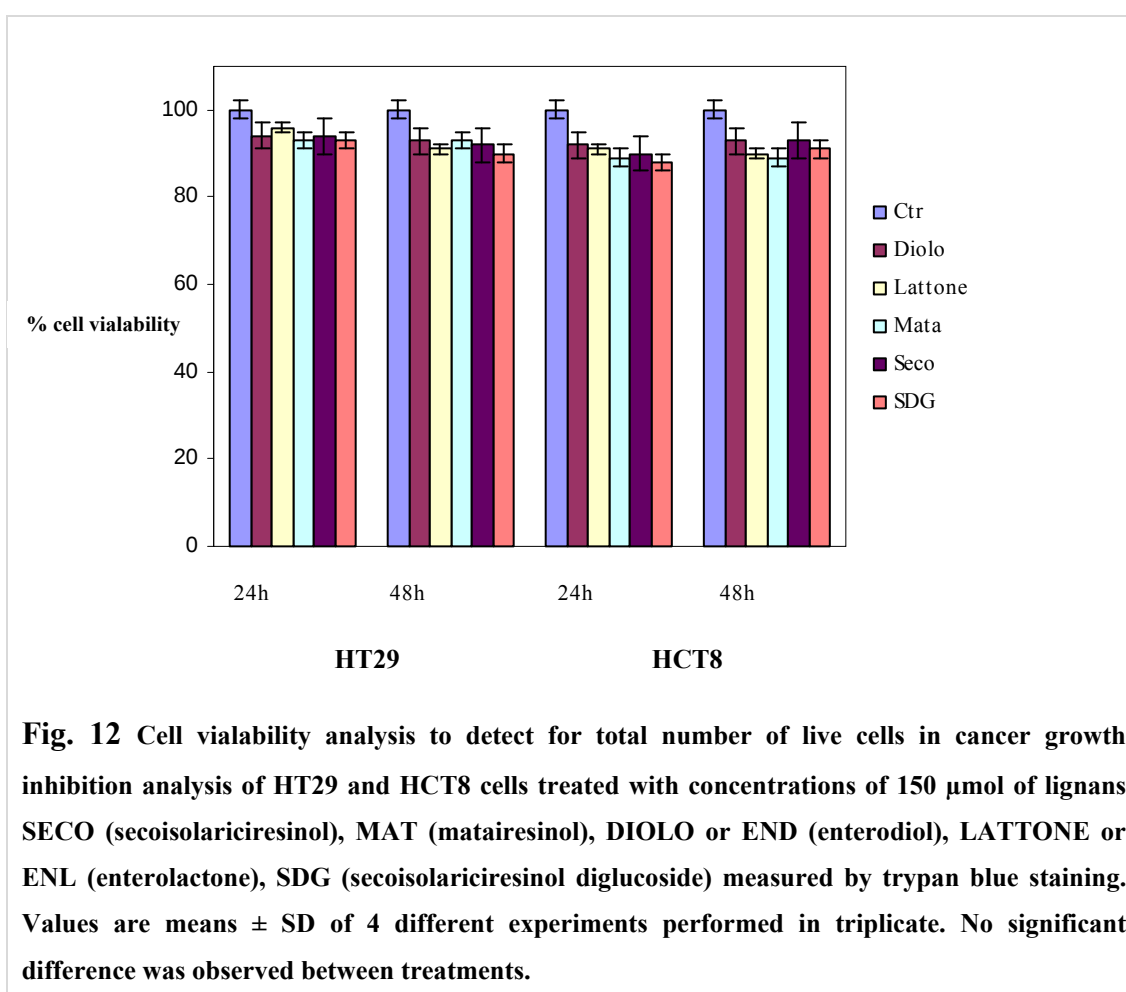


Fig. 12 Cell viability analysis to detect for total number of live cells in cancer growth inhibition analysis of HT29 and HCT8 cells treated with concentrations of 150 μ mol of lignans SECO (secoisolariciresinol), MAT (matairesinol), DIOLO or END (enterodiol), LATTONE or ENL (enterolactone), SDG (secoisolariciresinol diglucoside) measured by trypan blue staining. Values are means $\pm$ SD of 4 different experiments performed in triplicate. No significant difference was observed between treatments.

6.3 Cell Cycle

Cells HT29 and HCT8 were cultured with lignans SECO (secoisolariciresinol), MAT (matairesinol), END (enterodiol), ENL (enterolactone), SDG (secoisolariciresinol diglucoside) at 150 $\mu\text{mol/L}$ for 24 and 48 h and cell cycle change was measured through use of FACScalibur flow cytometer. DNA content analysis is based on the incorporation of propidium iodide into DNA and the resultant fluorescence is a measure of the relative proportion of cells in the various stages of the cell cycle. Figure 13 shows the percentage of cells HT29 and HCT8 in G1 phase after treatment with lignans at 150 $\mu\text{mol/L}$ at various times of exposure. DNA flow cytometry profiles indicated that G1-phase was significantly ($P \leq 0.05$) increased with lignans treatment. As the percentage of cells in G1-phase increased, the percentage of cells at both S and G2/M phases decreased correspondingly (data not shown). Percentage of cells in G1-phase was compared to vehicle control and is represented as mean $\pm$ SD from 4 independent experiments performed in triplicate. Cell cycle arrest may trigger the DNA repair machine, leading to apoptosis and this would be the mechanism of action. This could be in agreement with the work of Hausott et al., 2003. Our experiments, however, indicated that the treatment did not affect apoptosis in adherent cells compared with the vehicle (results and data not shown).

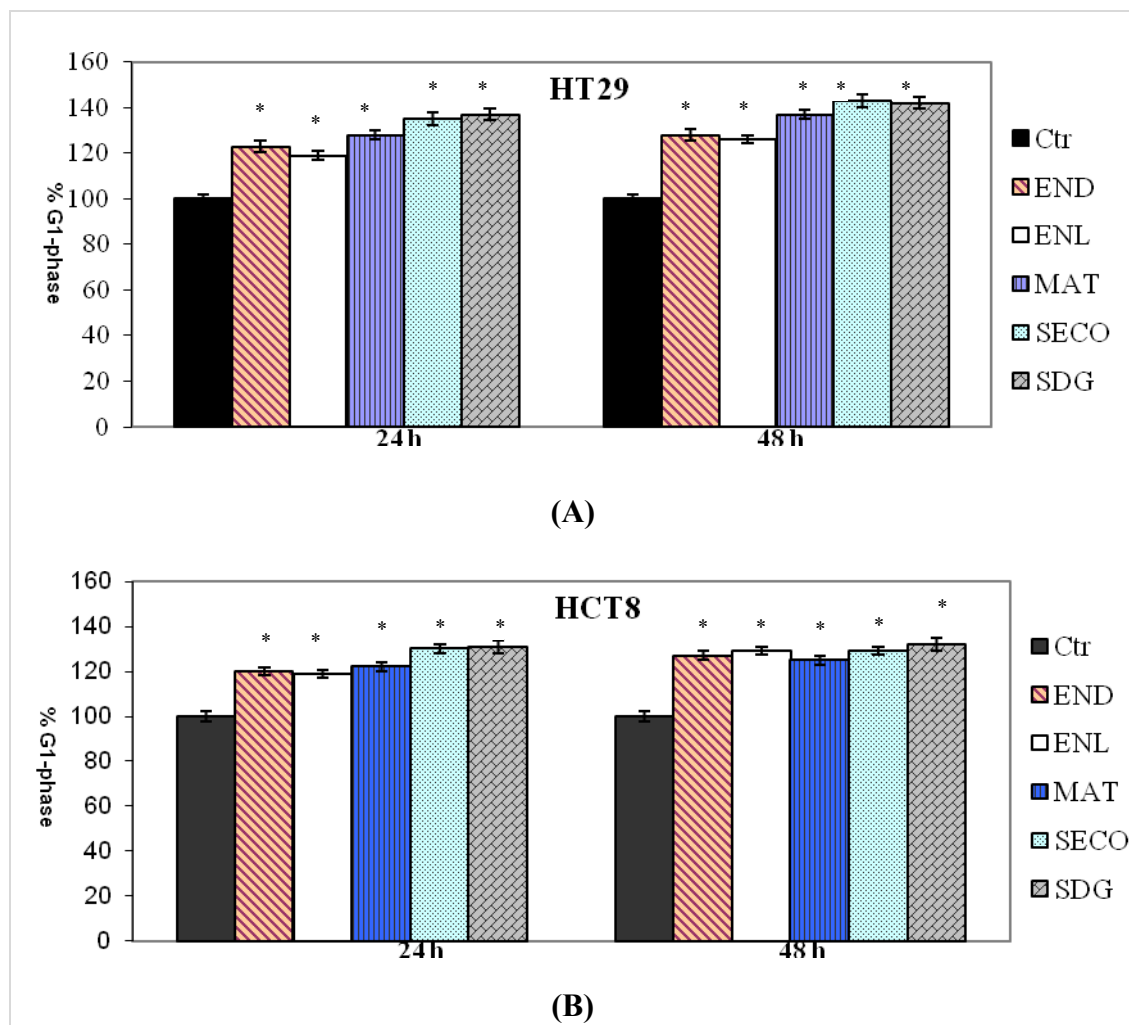


Fig. 13 Effects of 150 $\mu\text{mol/L}$ Lignans on cell cycle arrest in HT29 (A) and HCT8 (B) cells at G1 phase. Values are means $\pm$ SD of 4 different experiments, results are significant $P \leq 0.05$ (*).

6.4 Protein levels

The effect of lignans SECO (secoisolariciresinol), MAT (matairesinol), END (enterodiol), ENL (enterolactone), SDG (secoisolariciresinol diglucoside) on cell cycle regulatory molecules that are operative in the G1 phase of cell cycle is examined. Eukaryotic cellular proliferation is regulated by expression and sequential activation of cell cycle dependent cyclins, cyclin dependent kinases (CDKs) and CDK inhibitors (CKIs). Cyclins molecules regulate the progression of each phase of cell cycle by associating with corresponding phase specific CDKs. G1 to S cell cycle progression is controlled by several CDK complexes, including cyclinD1/CDK4 and cycline/CDK2. Cyclin D isoforms associate with CDK4 and CDK6 and this association leads to activation of CDK4 and CDK6 which help maintaining and progressing through the early G1 phase of the cell cycle. The activities of these complexes, are dependent on the balance of cyclins and CDK inhibitors (CKI), such p21 and p27. In this experiments, we decided to observe the behaviour of cyclin D1 and p-21 proteins. The expression of p21 and cyclin D1 in HT29 and HCT8 cells at 24 h as times of exposure was investigated in lignans (150 μ mol/L) treated and untreated cells by Western blot analysis. Figure 14 show the level of cyclin D1 was lower in cells treated with lignans 150 μ M in HT29 and HCT8 cells in relation to the control sample (GAPDH, glyceraldehyde-3-phosphate dehydrogenase expression was used as a control for equivalent lane loading and transfer efficiency) while P-21 protein level resulted increased in cells treated with lignans, especially in HT29 cells. In order to obtain a semi-quantitative value for the protein expression of p21 and cyclin D1 we measured the relative optical density (densitometry) of this proteins targets and GAPDH bands. The ratios of p21 and Cyclin D1 to GAPDH protein expression revealed that cells treated with 150 μ M lignans for 24 h showed decrease in cyclin D1 and increase in P-21 proteins level both compared to the untreated control (Figure 15). The induction of P-21 proteins expression were generally associated with the inhibition of cell growth.

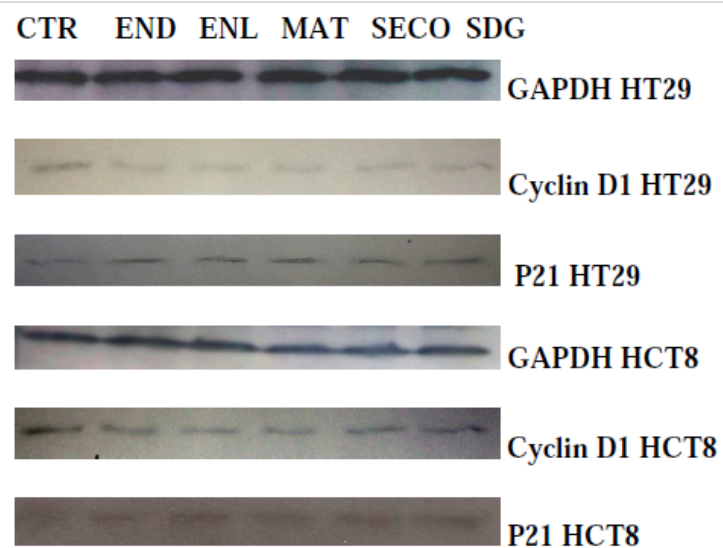
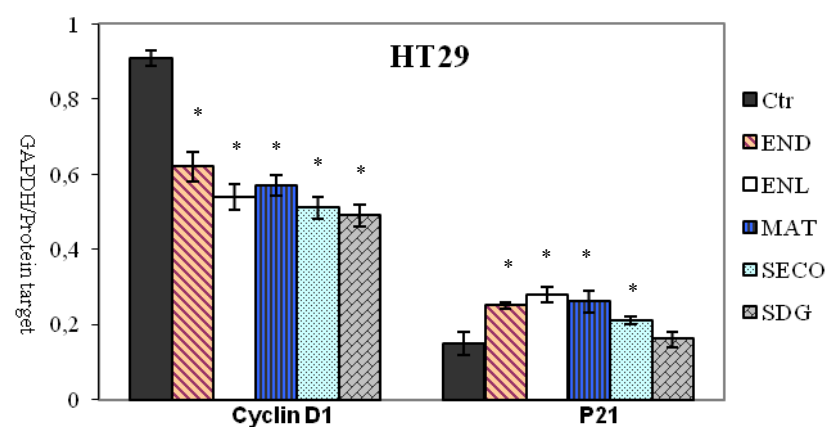
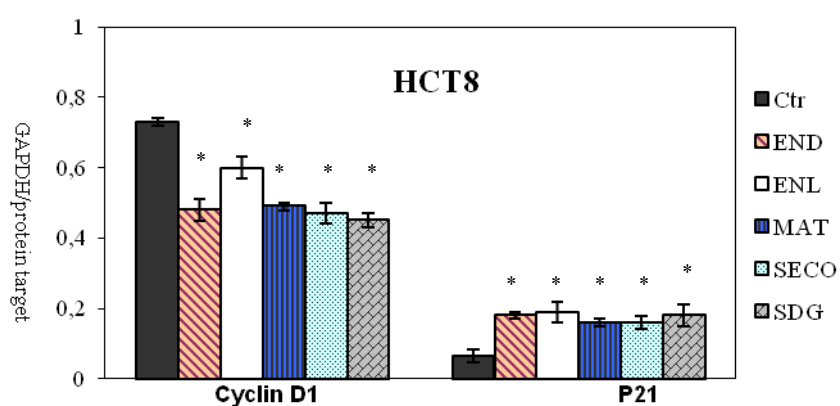


Fig. 14 Effects of 150 $\mu\text{mol/L}$ Lignans for 24 h on cyclin D1 and P21 protein levels in HT29 and HCT8 cells. Western blot analysis of HT29 and HCT8 cells for Cyclin D1, P21 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) protein levels (data from a representative experiment is shown from a total of four independent experiments) was performed with indicated antibodies. Protein loading was normalized based on GAPDH expression.



(A)

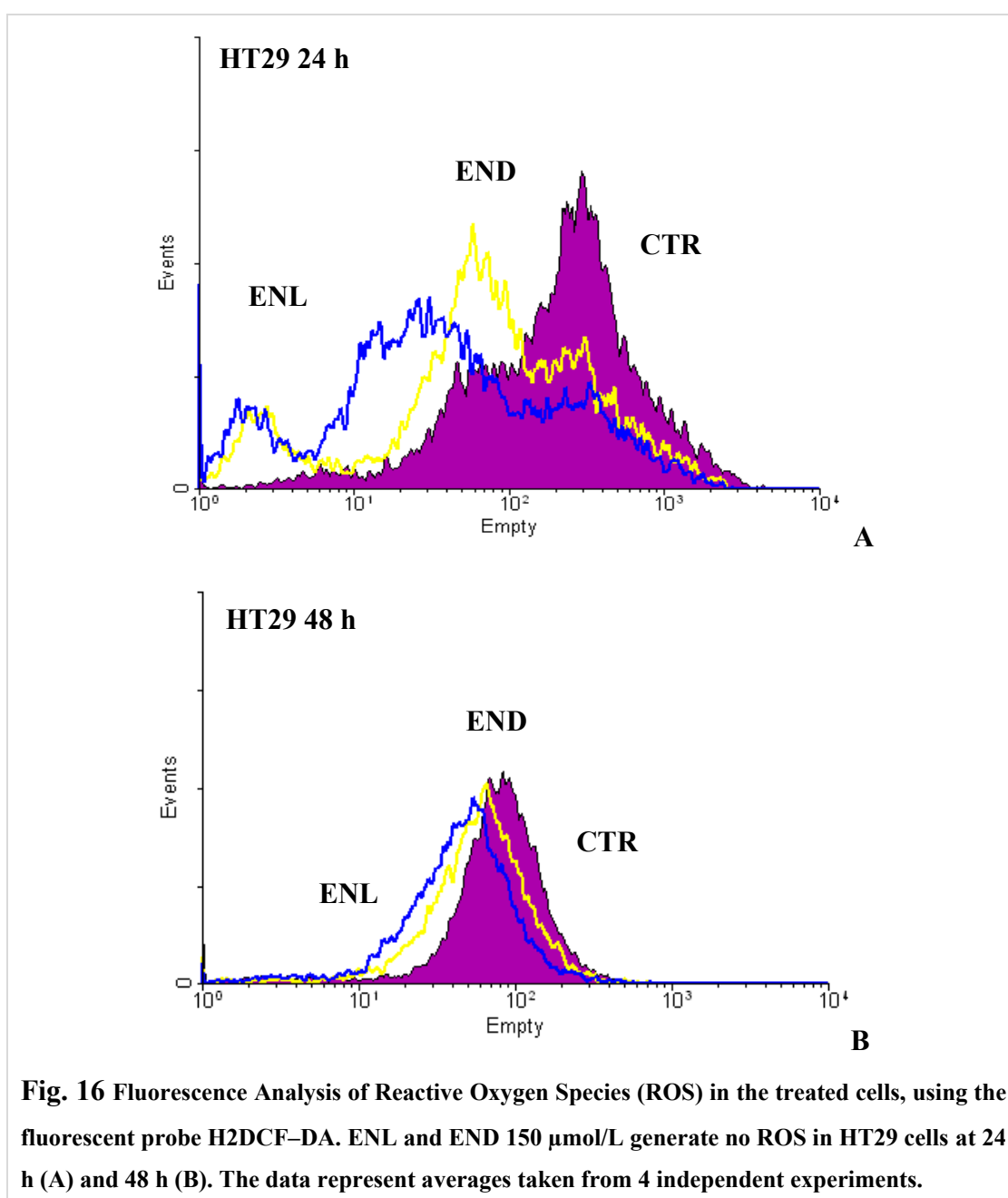


(B)

Fig. 15 Densitometric semi-quantification (band intensities) of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) protein expression on protein target. Effects of 150 $\mu\text{mol/L}$ lignans on cyclin D1 and P21 protein levels in HT29 and HCT8 cells. Values are means $\pm$ SD of 4 different experiments, results are significant $P \leq 0.05$ (*).

6.5 Analysis ROS generation

The analysis of intracellular ROS (reactive oxygen species) level with a probe of H2DCF-DA (2'-7'-dichlorofluorescein di-acetate) in treated (lignans ENL and END 150 $\mu\text{mol/L}$) HT29 and HCT8 cells showed a reduced concentration of ROS especially after 24 h compared to 48 h (Fig. 16 and Fig. 17 respectively). The lesser effect at 48 h is perhaps due to the short emivita of this antioxidant. In addition the HCT8 cells show higher oxidative intracellular status because their different state of differentiation respect to HT29 cells (Fig. 18).



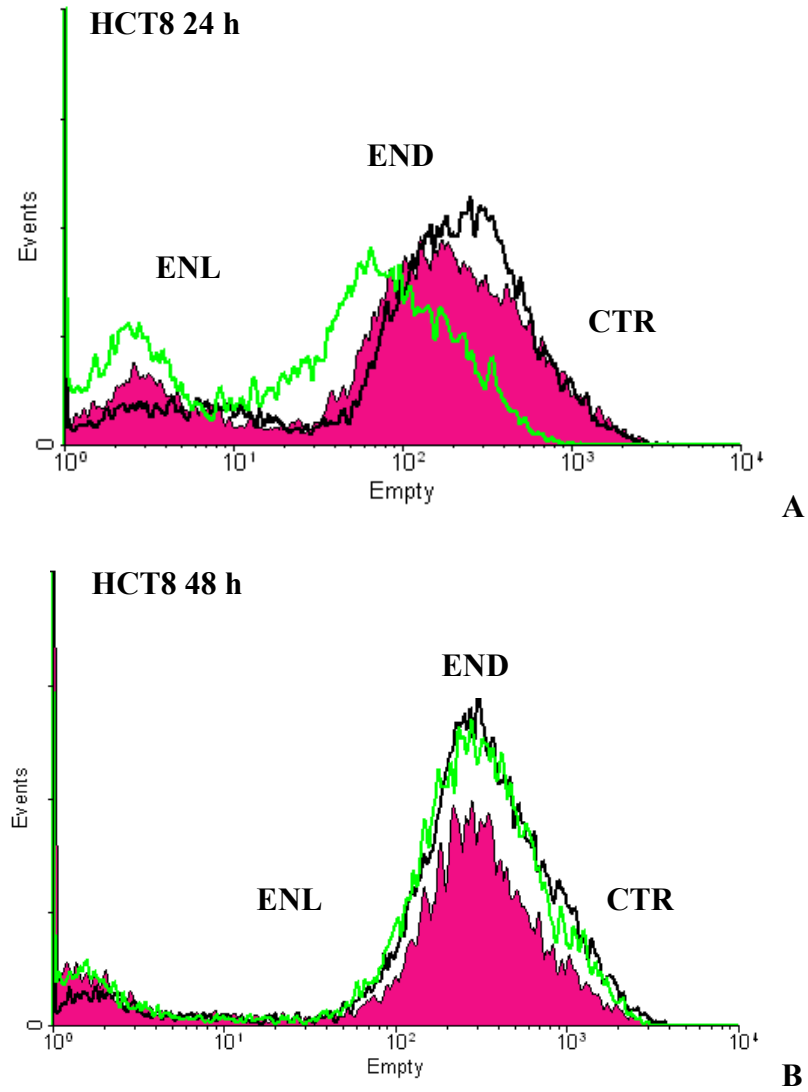


Fig. 17 Fluorescence Analysis of Reactive Oxygen Species (ROS) in the treated cells, using the fluorescent probe H2DCF-DA. ENL and END 150 $\mu\text{mol/L}$ generate no ROS in HCT8 cells at 24 h (A) and 48 h (B). The data represent averages taken from 4 independent experiments.

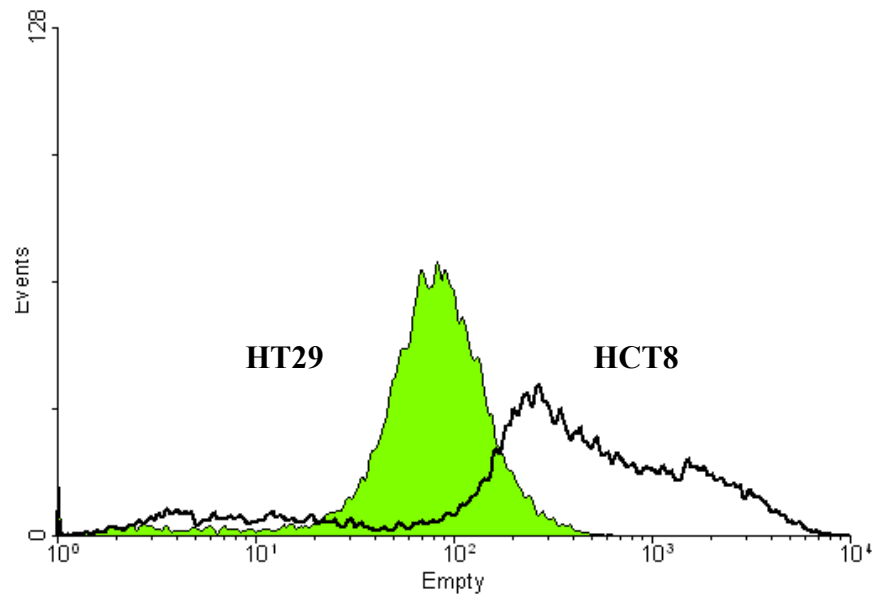


Fig. 18 Fluorescence Analysis of Reactive Oxygen Species (ROS) in untreated cells, using the fluorescent probe H2DCF-DA. HCT8 cells show higher oxidative intracellular status respect to HT29 cells at 24 h. The data represent averages taken from 4 independent experiments.

6.6 Buckwheat sprouts

Sprouting is a simple way to obtain a product with highly enhanced antioxidative capacity coming most likely from rapidly biosynthesized low molecular antioxidants. Buckwheat sprouts have a long and white hypocotyls (Fig. 19) that arrive at 12-15 cm long and 0.9-1 mm in diameter. Buckwheat sprouts had light yellow colored cotyledons and a bright-white colored hypocotyls. It was observed a different color of cotyledons and hypocotyls between common buckwheat (*Fagopyrium esculentum* Möench) and tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn.) sprouts and a different germination rate. The first kind of sprouts are dark the second light and the germination rate was 70% and 40% respectively. The pericarp shedding rate is one of the important characteristics of buckwheat sprouts because the pericarp attached to the buckwheat sprouts make it difficult to eat directly as a fresh vegetable. Pericarp shedding started at 5 days after seeding and the shedding rate was approximately 85% at 8 days after seeding. The water supply system as a spray was more effective than any other method in order to accelerate the shedding of buckwheat pericarp during germination. By using a pilot production system, approximately 259 gr of buckwheat sprouts could be harvested from 1 g of seed. Furthermore was observed a high germination ratio and obtained the buckwheat sprouts that had their pericarps completely removed. Fresh and dry weights of freshly sprouts harvested were determined and the variation in biomass and water were measured. Table 3 shows the variation during the oven drying process. % and g of weight loss biomass of sprouts, water content in % and g and loss water content of harvest was detected during process desiccation. The process lead to a yield of 15 g of dry matter (powder) after oven drying from 259 gr of initial quantity of harvest sprouts. In order to evaluate the possibility to use powder of buckwheat sprouts as food additional ingredient rich in bioactive compounds we analyze the composition of Common buckwheat seeds (*Fagopyrium esculentum* Möench) and tartary buckwheat seeds (*Fagopyrum tataricum* (L.) Gaertn.) and sprouts after cultivation of buckwheat sprouts.



A



B

Figure 19 Hydroponic culture of Buckwheat sprouts (produced in Department for Innovazione nei sistemi Biologici, Agroalimentari e Forestali DIBAF). Image (A) shows sprouting after 8 days after seeding. Picture B shows yield of 15 g of dry matter after oven drying from 259 gr of initial quantity of harvest sprouts.

Time of relief (Hours)	1° day desiccation					2° day desiccation					3° day final relief		
	12,5	13,5	14,5	15,5	17,28	10,3	11,3	12,3	17	18	10	11	12
Weight biomass (g)	259	250	241	232	213	93	87	79	70	58	15	15	15
Water content (g)	244	235	226	217	198	78	72	64	55	43	0	0	0
% water content	100	96	93	89	81	32	30	26	23	18	0	0	0
% Weight loss biomass	0	3	7	10	18	64	66	69	73	78	94	94	94
% loss water content	0	4	7	11	19	68	70	74	77	82	100	100	100

Table 3 Biomass and water content sprouts changes during the drying process.

6.7 Bioactive compounds contents of buckwheat sprouts and seeds

The TPC (Total phenolic contents) of buckwheat seed and sprouts extracts was determined (according to the method of Shahidi and Naczki, 1995) using the Folin–Ciocalteu reagent and are calculated from a standard curve using a linear range of concentration of ferulic acid. Three individual experiment were performed. Values are expressed as mean $\pm$ standard deviation. Table 2 shows the results obtained analyzing total phenolic contents (TPC) of common buckwheat (*Fagopyrium esculentum* Möench) and tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn.) seeds and sprouts. Polyphenols contents are different in different part of the plant. TPC change in different parts of the plant: they are very high in sprouts in comparison to grounded dry seeds. Tartary buckwheat seeds and sprouts show higher content of TPC than Common buckwheat.

Sample seeds	TPC (mg/gr d.m.)	Sample sprouts	TPC (mg/gr d.m.)
Common buckwheat (<i>Fagopyrium esculentum</i> Möench)	2,78 $\pm$ 0,02	Common buckwheat (<i>Fagopyrium esculentum</i> Möench)	31,29 $\pm$ 0,4
Tartary buckwheat (<i>Fagopyrum tataricum</i> (L.) Gaertn.)	8,32 $\pm$ 0,01	Tartary buckwheat (<i>Fagopyrum tataricum</i> (L.) Gaertn.)	46,56 $\pm$ 0,3

Table 4 TPC (Total phenolic contents) of buckwheat seed and sprouts. Three individual experiment were performed. Values are expressed as mean $\pm$ standard deviation.

§ 7. DISCUSSION

“Functional foods”, “nutraceuticals” or “pharmaconutrients” are all terms, which indicate nutrients or nutrient enriched foods that can prevent or treat diseases (Hardy, 2000). These foods and food components represent the fastest growing segment in food industry. In this contest, in the last decade, whole cereal, buckwheat, soy, flaxseed and their products are notified the most common source based functional food and nutraceuticals (Andlauer *et al.*, 2002) rich in bioactive compounds like phytoestrogens. Phytoestrogens are a group of biologically active plant substances with a chemical structure that is similar to that of estradiol and have both estrogenic and anti-estrogenic effects (presence of a phenolic ring that allows them to bind to the estrogen receptor (ER) mimicking the effects of mammalian estrogens). The major classes of phytoestrogens are isoflavones, lignans and coumestans. Nutritional intervention studies in humans, animals and test *in vitro* suggest that dietary phytoestrogens have protective effects against cardiovascular disease, hyperlipidemia, osteoporosis and cancer. Indeed epidemiological studies correlate high concentrations of lignans in body fluids, due to dietary intake, with a low incidence of hormone-dependent tumors, in particular breast, ovary and prostate neoplasia and with non hormone-dependent cancer such as colon cancer (Heinonen S, *et al.* 2001). The mechanisms on the basis of this observations seems to be linked to the fact that END and ENL significantly inhibited the growth of human colon tumor cells and that the proliferation of MCF-7 breast cancer cells induced by E2 (17 β -estradiol) was inhibited by ENL. Furthermore ENL is capable of inducing quinine reductase, in Colo205 cells through an enzymatic mechanism. Moreover other studies demonstrated that treatment of human colon cancer SW480 cells with lignans (SDG vs. its metabolite ENL) results in a dose and time dependent inhibition of cancer cell growth. ENL is also shown to induce apoptosis and inhibit growth in human colon cancer cell line, colo-201. In four other human colon tumor cell lines: LS174T, CaCo-2, HCT-15 and T-84, ENL and END at 100 μ M concentration reduced cell proliferation. Based on this research, in the first part of this thesis, we report the effects *in vitro* of the different phytoestrogens type lignans SECO, MAT, END, ENL and SDG on proliferation of human colon cancer cells lines HT29 and HCT8. The results showed that lignans were able to inhibit proliferation and to

induce the arrest of cell cycle in the G1-phase on both cellular lines but with more evidence for HCT8 cells line. The human colonic cancer cells HT29 and HCT8 are respectively from rectal tract and ileo-cecal tract of the gastrointestinal system of unhealthy humans. Moreover the HT29 cells are in the first state of differentiation, whereas HCT8 cells are in the second state of differentiation. This means that HT29 cells are more similar to the original tissue than the other cells and so we can justify the different results in various experiments. The reduction in cell numbers did not seem to be due to toxicity because cell viability was not changed by any of the treatments, as shown by trypan blue assay. The concentrations of lignans used are high relative to plasma levels in humans or animals (usually in the nanomolar range). The high concentration of metabolite lignans used in this study might be achievable in the enterocytes of the gut lumen where lignans are hydrolyzed by intestinal flora and then absorbed (range μM) while the levels of lignans metabolites in plasma are low because of their efficient enterohepatic circulation (Hausott *et al.*, 2003). This is the first report to document that lignans, precursor and metabolite, inhibit proliferation and induce the arrest of cell cycle in HT29 and HCT8 cells. It is well known that G1 to S cell cycle progression is controlled by several CDK complexes, including cyclinD1/CDK4 and cyclinE/CDK2. The activities of these complexes, are dependent on the balance of cyclins and CDK inhibitors (CKI), such p21 and p27. In order to study the mechanism of action underlying this observation we studied the behaviour of cyclin D1 and p-21 protein. The result reported showed that cyclin D1, tested by western blot analysis, showed a down-modulation especially in HT29 and HCT8 cells treated with SDG while p-21 protein level resulted increased in cells treated with lignans. Our experiments, however, indicated that the treatment did not affect apoptosis in adherent cells compared with the vehicle (results and data not shown). Considering that lignans seems to be antioxidant compounds we measured the intracellular ROS (reactive oxygen species) level, with a probe of H2DCF-DA, in treated HT29 and HCT8 cells. The analysis showed a reduced concentration of ROS especially after 24 h compared to 48 h. The lesser effect at 48 h is perhaps due to oxidation of self metabolites. In addition the HCT8 cells show higher oxidative intracellular status because their different state of differentiation respect to HT29 cells as above described. Even though more research needs to be done to complete our knowledge about their antioxidant and anti-tumor activity these preliminary findings suggesting a potential use of plant lignans as ingredient for functional food.

Thus, in the second part of this thesis, we report the procedure for growth and characterization of buckwheat seeds and sprouts, as preliminary step for a future setting up of a proof-project for formulation of functional food containing significant levels of biologically active components like phytoestrogens. For this aim hydroponic culture of Buckwheat sprouts was produced (with the help of Prof. Mazzuccato of the Department for Innovazione nei sistemi Biologici, Agroalimentari e Forestali DIBAF). After 8 day of growth the sprouts were harvested and fresh and dry weights were determined and the variation in biomass and water were measured. The variation during the oven drying process (% and g of weight loss biomass of sprouts, water content in % and g and loss water content of harvest was detected during process desiccation) lead to a yield of 15 g of dry matter after oven drying from 259 gr of initial quantity of harvest sprouts. Rutin, quercetin and other polyphenols content in seeds and other part of this pseudocereal, depends on variety and growing conditions. For this reason is important to analyze every sample of buckwheat before to do experiments. The results obtained analyzing total phenolic contents (TPC) of buckwheat seeds and sprouts showed change of contents in seeds and sprouts. TPC was very high in sprouts in comparison to grounded dry seeds. Tartary buckwheat sprouts and seeds show higher content of TPC than Common buckwheat. This is very interesting to use as raw source of functional food, tartary buckwheat sprouts, because its content of bio-active compounds is much higher than in common buckwheat, which has been traditionally utilized throughout the world. As this sprouts are consumed at the beginning of the growing phase, their nutrient concentration remains very high and studies show that maximum yields of most nutrient occurred on day 8 sprouting. Tartary buckwheat has the disadvantages of bitter taste, small seed size, and a hull that firmly adheres to the testa thus making de hulling extremely difficult. Biochemical composition and utilization of buckwheat sprouts as a valuable, functional vegetable has been reported but no studies showed interest for dried and powdered sprouts as additional ingredients in functional food. Hence buckwheat sprouts powdered could be an important raw material for new functional food production.

§ 8. CONCLUSIONS AND FUTURE PERSPECTIVES

This PhD research focused the attention on the biological effects of phytoestrogens (in particular of lignans) *in vitro* and report procedure for selection and characterization of bioactive compounds from buckwheat seeds and sprouts. The research produced promising results which open horizons on the possibility to produce innovative food that provide health benefit using, for the first time, powder of Tartary buckwheat sprouts (*Fagopyrum tataricum* (L.) Gaertn.) rich in bioactive compounds. Further studies are direct to characterize the phytoestrogens lignans in buckwheat seeds and dried sprouts. In addition as regard future prospective, the systemic *in vivo* influence (oxidative status, immune response) of different lignans on experimental animals fed with sprouts powder and/or fed with extraction yields from buckwheat sprouts will be evaluate.

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§ 10. GLOSSARY

FUNCTIONAL FOOD: The term functional food was first introduced in Japan in the mid-1980s and refers to processed foods containing ingredients (a new ingredient(s) or more of an existing ingredient has been added to a food and the new product has a new function) that aid specific bodily functions in addition to being nutritious. "Functional foods", "nutraceuticals" or "pharmaconutrients" are all terms, which indicate nutrients or nutrient enriched foods that can prevent or treat diseases. The general category of functional foods includes processed food or foods fortified with health-promoting additives, like "vitamin-enriched" products. Functional foods are an emerging field in food science due to their increasing popularity with health-conscious consumers and the ability of marketers to create new interest in existing products.

PHYTOESTROGENS: Phytoestrogens are natural estrogenic compounds found in a diverse number of plants; these compounds have similar structures to mammalian estrogens and have both estrogenic and anti-estrogenic effects (presence of a phenolic ring that allows them to bind to the estrogen receptor (ER) mimicking the effects of estrogen). Phytoestrogens have been categorized according to their chemical structures as isoflavones, lignans and coumestans. Evidence is accruing that phytoestrogens may have protective action against diverse health disorders. The foods with the highest relative phytoestrogen content were nuts and oilseeds, followed by soy products, cereals and breads and legumes.

LIGNANS: Lignans, a type of phytoestrogens, are constituents of many plants and form the building blocks for the formation of lignin in the plant cell wall. They are ubiquitous in the woody portions of plants. Plants (poly)phenols like lignans are derived from phenylalanine units, and they have in common at least one aromatic ring structure with hydroxyl group(s). They are found in the woody portions of plants, the seed, coat of seeds, and the bran layer in grains. They are estrogen-like chemicals and also act as antioxidants. Some examples of lignans are pinoresinol, podophyllotoxin, steganacin, lariciresinol, secoisolariciresinol, matairesinol, hydroxymatairesinol, syringaresinol and sesamin. Much data suggests that lignans contribute to cancer and other disease prevention in various cell lines, in vivo and in epidemiological studies.

MAMMALIAN LIGNANS: The two most important are *trans*-2,3-bis(3-hydroxy-benzyl)- γ -butyrolactone (enterolactone, ENL) and 2,3-bis(3-hydroxybenzyl)butane-1,4-diol (enterodiol, END). They are the major lignans present in serum, urine, bile and seminal fluids of humans and animals. Because these two compounds are produced in animals as opposed to plants they are usually termed the mammalian lignans to distinguish them from lignans from plants (differ from plant-derived lignans in possessing phenolic hydroxy groups only in the *meta*-position of the aromatic rings). After ingestion, some plant lignans can be converted to the enterolignans, enterodiol (END) and enterolactone (ENL) by the intestinal microflora, and absorbed into the body.

COLORECTAL CANCER: Colorectal cancer, commonly known as bowel cancer, is a cancer from uncontrolled cell growth in the colon, rectum, or appendix. Symptoms typically include rectal bleeding and anemia which are sometimes associated with weight loss and changes in bowel habits. Most colorectal cancer occurs due to lifestyle and increasing age with only a minority of cases associated with underlying genetic disorders. Screening (sigmoidoscopy or colonoscopy) effective at decreasing the chance of dying from

colorectal cancer and is recommended starting at the age of 50 and continuing until a person is 75 years old. Cancers that are confined within the wall of the colon are often curable with surgery while cancer that has spread widely around the body is usually not curable and management then focuses on extending the person's life via chemotherapy and improving quality of life. Colorectal cancer is the third most commonly diagnosed cancer in the world, but it is more common in developed countries. It is estimated that worldwide, in 2008, 1.23 million new cases of colorectal cancer were clinically diagnosed, and that it killed 608,000 people.

BUCKWHEAT: The name “buckwheat” (a non-glutinous pseudo-cereal Polygonaceae) comes from the Anglo-Saxon words *boc* (beech) and *whoet* (wheat) because the seed resembles a small beech nut. Buckwheat has gained an excellent reputation for its nutritious qualities in the human diet. Its renewed popularity stems from its many bioactive components, which have been shown to provide various health benefits much sought after in natural foods. It positively affects the human organism biologically if consumed as vegetables (for example low glycemic index and reduced capillary fragility, bloody pressure) without the necessity of adding any other components. Buckwheat is uniquely rich in proteins (12-15%) and essential amino acids, such as lysine (5-7%), that are deficient in major cereal crops, but also contains an abundance of lipids, fibres, minerals (zinc, manganese, selenium, iron, phosphorus, and copper), and vitamins (B1 and B2). The buckwheat sprouts are outstanding sources of protein, vitamins and minerals and they contain such in the respect of health-maintaining important nutrients. Buckwheat sprouts have a significantly higher antioxidant activity than seeds. Buckwheat flour, groats and seeds are used for a wide variety of dishes. For example the flour is mixed with wheat flour for the production of buckwheat noodles called ‘soba noodles’ in Japan or pasta like Pizzoccheri in Italia.

CELLULAR PROLIFERATION: Increase in the number of cells as a result of cell growth and cell division. The biology of cell division, differentiation, and apoptosis is exceedingly similar in both normal and cancer cells. The cancer cell differs from its normal counterpart in that it is aberrantly regulated. Cancer cells generally contain the full complement of biomolecules that are necessary for survival, proliferation, differentiation, cell death, and expression of many cell-type specific functions. Failure to regulate these functions properly, however, results in an altered phenotype and cancer. The rate of cell proliferation within any population of cells depends on the rate of cell division, the fraction of cells within the population undergoing cell division (growth fraction), and the rate of cell loss from the population due to terminal differentiation or cell death.

CELL CYCLE: The process a cell goes through each time it divides. The cell cycle consists of a series of steps during which the chromosomes and other cell material double to make two copies. The cell then divides into two daughter cells, each receiving one copy of the doubled material. The cell cycle is complete when each daughter cell is surrounded by its own outer membrane. Also called mitotic cycle. The cell division cycle can be divided into two functional phases, S and M phases, and two preparatory phases, G1 and G2. S phase is defined as the phase in which the DNA is replicated. Under normal circumstances, the time it takes a typical human cell to complete S phase is about 8 hours and is invariant. Fully replicated chromosomes are segregated to each of the two daughter nuclei by the process of mitosis during M phase. The length of M phase is about 1 hour and is also normally invariant. G1 phase precedes S phase, whereas G2 phase precedes M phase. G1 and G2

phases are required for the synthesis of cellular constituents needed to support the following phase and ultimately to complete cell division. In mammalian cells, the length of G2 phase is about 2 hours. The length of G1 phase is highly variable and can range from about 6 hours to several days or longer. The varying length of G1 phase accounts for most of the difference in rate of cell division between different cell types or between cells growing under different conditions.

ANTIOXIDANT: A substance that protects cells from the damage caused by free radicals (unstable molecules made by the process of oxidation during normal metabolism). Free radicals may play a part in cancer, heart disease, stroke, and other diseases of aging. Antioxidants include beta-carotene, lycopene, polyphenols as lignans, flavonoids, vitamins A, C, and E, and other natural and manufactured substances.

POLYPHENOLS: Polyphenols are a structural class of natural, synthetic, and semisynthetic organic chemicals characterized by the presence of large multiples of phenol structural units. The number and characteristics of these phenol structures underlie the unique physical, chemical and biological (metabolic, toxic, therapeutic, etc.) properties of particular members of the polyphenol class. The term polyphenol appears to have been in use since 1894. From about 500 million years ago, freshwater and terrestrial plants slowly optimized the production of “new” endogenous antioxidants, such as ascorbic acid (vitamin C), polyphenols (including flavonoids), tocopherols, etc. Consuming dietary polyphenols may be associated with beneficial effects in higher animal species. The main source of polyphenols is dietary, since they are found in a wide array of phytochemical-bearing foods. For example, honey, most legumes, fruit and vegetables, red wine, chocolate, white tea, green tea, olive oil, argan oil, bee pollen and many grains are sources. Ingestion of polyphenols occurs by consuming a wide array of plant foods. The regulation theory considers a polyphenol antioxidant’s ability to scavenge free radicals and up-regulate certain metal chelation reactions. Various reactive oxygen species, such as singlet oxygen, peroxynitrite and hydrogen peroxide, must be continually removed from cells to maintain healthy metabolic function. Diminishing the concentrations of reactive oxygen species can have several benefits possibly associated with ion transport systems and so may affect redox signaling.

ABBREVIATIONS

AC	aberrant crypt	ILs	interleukin
ACF	aberrant crypt foci	LAR	lariciresinol
AOM	azoxymethane	LDL	low density lipoprotein
APC/Apc	adenomatous polyposis coli	LOH	loss of heterozygosity
ATCC	American Type Culture Collection	LPS	lipopolysaccharide
BMI	body mass index	MAT	matairesinol
BrdU	5-bromo-2'-deoxyuridine	MIN	multiple intestinal neoplasia
CFU	colony-forming unit	N-MNU	methyl-N-nitrosourea
CL	chemiluminescence	NK	natural killer
CNS	central nervous system	NO	nitric oxide
COX	cyclooxygenase	NOC	N-nitroso compounds
DMBA	dimethylbenz(a)anthracene	NSAIDs	anti-inflammatory drugs
DMSO	dimethylsulfoxide	PGE2	prostaglandin
DW	dry weight	PAHs	polycyclic aromatic hydrocarbons
E2	17β-oestradiol	PINO	pinoresinol
END	enterodiol	PBS	phosphate Buffer Saline
ENL	enterolactone	POD	peroxidase
ER	oestrogen receptor	PI	iodure propidium
FAP	familial Adenomatous Polyposis	ROIs	reactive oxygen intermediates
FCS	foetal bovine serum	SECO	secoisolariciresinol
FRAP	ferric Reducing Antioxidant Power	SERMs	modulators
GC-MS	gas chromatography-mass spectrometry		selective estrogen receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	ROS	reactive oxygen species
HCA s	heterocyclic amines	SHBG	sex hormone binding globulin
HDL	high density lipoprotein	SD	standard deviation
HNPCC	hereditary non-polyposis colorectal cancer or lynch syndrome	SDG	secoisolariciresinol diglycoside
HPLC	high-performance liquid chromatography	SDS	sodium dodecil sulphate
H2DCF-DA	2'-7'-dichlorofluorescein diacetate	SYR	syringaresinol
HRT	hormone replacement therapy	TBARS	thiobarbituric acidreactive substance
		TEAC	trolox-equivalent antioxidant activity
		TNF-α	tumor necrosis factor
		TC	triacylglycerol
		TPC	total phenolic contents

APPENDIX

List of published papers, poster communications included.

- Molinari, R., Manzi, L., **Ricci, S.**, D'Aquino, M., Tomassi, G., Papeschi, Merendino, N. Diet rich in whole wheat improve redox status and enhance immune responses in rats. J. Food and Agriculture immunology. 2009.
- 14th Workshop on the Development in the Italian PhD Research on Food Science Technology and Biotechnology, University of Sassari, Oristano (Italy), 16-18 Settembre, 2009
- 15th Workshop on Developments in the Italian PhD Research in Food Science and Technology, University of Napoli I – Federico II, Portici, 15-17 September, 2010
- 16th Workshop on Developments in the Italian PhD Research in Food Science and Technology, University of Milan and Piacenza, Lodi, 21-23 September, 2011

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