Anti-Cancer Effects of Bioactive Compounds from Rose Hip Fruit in Human Breast Cancer Cell Lines

Carotenoids, Triterpenes, and Ascorbate Evaluated Singly or in Combination

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Cover: Rose hip fruits, TNF-α induced NF-κB translocated to the nucleus, and inhibited TNF-α induced NF-κB translocation (photo: L. Zhong). Molecular structure (L. Zhong). Pattern design (X. Qiao).

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Abstract

Rose hips have long been used in human diets as a food ingredient and supplement. Their multiple medical properties, which have been attributed to their abundant carotenoid composition, have attracted widespread scientific attention. This thesis examined the carotenoid composition in rose hips from five rose species. The anti-cancer effect of different carotenoid fractions from rose hips was investigated in human breast cancer cell lines, using the natural variation in carotenoid content in hips from different rose species. Based on the results obtained, representative single carotenoids were selected for further investigation.

A rapid, effective method was developed using a HPLC-DAD-APCI$^+$-MS system for carotenoid identification and quantification in rose hips. Twenty-one carotenoids, including 11 xanthophylls and 10 carotenes, were detected in saponified extract and 23 carotenoid esters in unsaponified extract from hips of the five rose species. Three fractions were subsequently isolated from total carotenoid extract of rose hips and their anti-proliferative activities were investigated in human breast cancer cell lines. Xanthophyll ester fraction was proved more potent than lycopene isomer or an undefined carotene fraction. *Rosa multiflora* Thunb. hips were the most effective, giving low IC_{50} values in MTT assay, and a unique xanthophyll ester pattern was found in this species.

When xanthophyll ester was combined with triterpenes in MTT assay, synergistic effects were found in the MCF-7 cancer cell line. A significant synergistic effect was also found on combining a 10 µM concentration of rose hip triterpene fraction with 50 µM ascorbate, which resulted in strong inhibition in cell proliferation in MCF-7 cell line, while normal-like cells MCF-10A were relatively undamaged.

Xanthophyll esters were verified to be more effective than free xanthophylls in MTT assay comparing lutein and zeaxanthin with their esters. The xanthophyll ester fraction from *R. multiflora* and individual xanthophyll esters were found to decrease cancer stem cell sub-populations and inhibit cell migration. Xanthophyll esters were suggested to affect breast cancer cells by a mechanism involving the NF-κB pathway.

Overall, these results support the beneficial health effects of rose hips and reveal potential to involve xanthophyll esters in future breast cancer therapy.

Keywords: rose hip, fruit, xanthophyll ester, *Rosa* sp., breast cancer, JIMT-1, MCF-7, MCF-10A, MTT assay, anti-proliferative, synergistic effect, cancer stem cell

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Dedication

To my family, friends and myself.

Smile . . . tomorrow will be worse.
Murphy's philosophy

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Zhong, L., Gustavsson, K.E., Oredsson, S., Głąb, B., Yilmaz, J.L. & Olsson, M.E. (2016). Determination of free and esterified carotenoid composition in rose hip fruit by HPLC-DAD-APCI^{+}-MS. *Food Chemistry* 210, 541-550.

II Zhong, L., Olsson, M.E., Huang, X., Gustavsson, K.E. & Oredsson, S. Cancer phenotype-specific effect of the xanthophyll ester fraction extracted from rose hips in a human breast cancer cell line. (Submitted)

III Zhong, L., Paulsen, F., Strand, D., Oredsson, S. & Olsson, M.E. Anti-cancer activities of lutein dimyristate and zeaxanthin dimyristate in human breast cancer cell lines and a breast epithelial cell line. (Manuscript)

IV Zhong, L., Oredsson, S. & Olsson, M.E. Effects of combining triterpenes, lutein dimyristate and ascorbate on cell proliferation in human breast cancer cell lines and a breast epithelial cell line. (Manuscript)

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The contribution of Lijie Zhong to the papers included in this thesis was as follows:

I Planned the experiment with the supervisors, performed the experimental work, analysed the chromatograms and wrote the manuscript with contributions from the co-authors.

II Participated in planning the experiment with the supervisors, performed the experimental work and data analysis and wrote the manuscript with contributions from the co-authors.

III Participated in planning the experiment with the supervisors, performed the experimental work and data analysis and wrote the manuscript with contributions from the co-authors.

IV Planned the experiment with the supervisors, performed all the experimental work and data analysis and wrote the manuscript with contributions from the co-authors.

Abbreviations

ALDH	Aldehyde dehydrogenase
APCI	Atmospheric pressure chemical ionisation
BA	Betulinic acid
CSC	Cancer stem cell
DAD	Diode array detector
HER2	Human epidermal growth factor receptor 2
HPLC	High performance liquid chromatography
IC_{25}	Concentration giving 25% inhibition
IC_{50}	Concentration giving 50% inhibition
MDR	Multidrug resistance
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NF-κB	Nuclear factor-κB
OA	Oleanolic acid
PBS	Phosphate-buffered saline
ROS	Reactive oxygen species
TCE	Total carotenoid extract
TF	Triterpene fraction
THF	Tetrahydrofuran
TNF-α	Tumour necrosis factor-α
UA	Ursolic acid

1 Introduction

1.1 Rose hips

Rose hips, also called rose haws or rose heps, are the fruit of the rose plant (Figure 1). The typical colour of rose hips is orange to red, but it ranges from dark purple to black in some species. Rose hips are commonly used as herbal tea and to make jam, jelly, marmalade, and rose hip wine. An oil is also extracted from the seeds. Rose hip soup (*nyponsoppa*) is an especially popular drink in Sweden. A traditional fruit brandy (*pálinka*) made from rose hips is popular in Hungary, Romania and other countries sharing an Austro-Hungarian history. Rose hips are also used as the central ingredient of *cockta*, the fruity-tasting national soft drink of Slovenia.

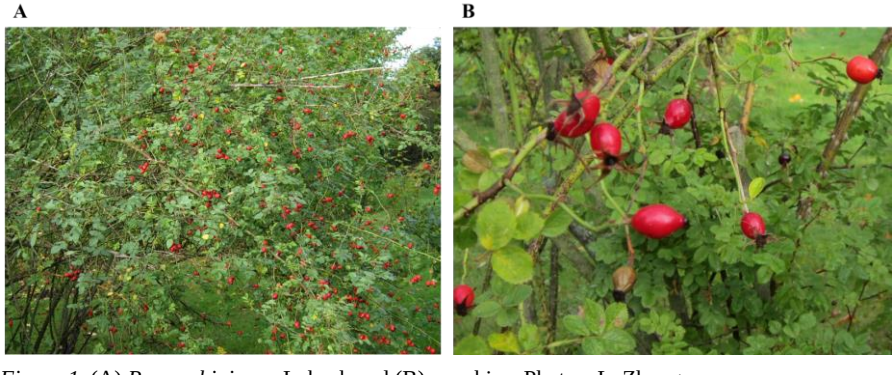

Figure 1. (A) *Rosa rubiginosa* L. bush and (B) rose hips. Photos: L. Zhong.

1.1.1 Origin, history and cultivation of the rose plant

Roses belong to the genus *Rosa*, in the family Rosaceae. Most species are native to Asia, with smaller numbers native to Europe, North America and north-west Africa. There are more than 100 species in the *Rosa* genus growing in temperate and subtropical climates of the Northern hemisphere. For over

4700 years, roses have been used mainly for ornamental purposes, but also for cosmetic and medical purposes (Gustavsson, 1998).

Rose bushes require relatively low levels of horticultural maintenance. The fruit is a potentially profitable crop for the food and nutraceutical industries. In the food industry, rose hips from dogrose (*Rosa canina* L.) have a long tradition of use in various types of foods around the world (Werlemark & Nybom, 2010). However, many of the rose hips used today for commercial products are still collected from the wild, as traditional rose breeding objectives have mainly focused on cut flower properties, including productivity, postharvest life and increased fragrance, but not on rose hip production (Gudin, 2000). The first commercial rose hip plantations in southern Sweden were established in the mid-1980s, primarily using superior selections mainly of the species *Rosa dumalis* Bechst. and *Rosa rubiginosa* L. which were chosen based on yield, disease resistance, fruit quality (*e.g.* high content of ascorbic acid, total antioxidants, high level of dry matter) and their suitability for mechanical harvesting (Uggla, 2004; Uggla & Nybom, 1996). Since the 1990s, Kovács and co-workers in Hungary have evaluated the physical and phytochemical characteristics of rose hips from a series of native Hungarian rose species for commercial rose hip production and future rose hip breeding (Kovács *et al.*, 2010; Kovacs *et al.*, 1999). However, commercial rose hip breeding has not been developed widely to date.

1.1.2 Health properties of rose hips

Rose hips contain abundant phytochemicals, including phenolics, flavonoids, tocopherols, fatty acids, and triterpene acids, with especially large amounts of ascorbate (vitamin C), and carotenoids (Zhong *et al.*, 2016; Wenzig *et al.*, 2008; Olsson *et al.*, 2004). Various phytochemicals in rose hips have antioxidant activity and can act as scavengers of reactive oxygen species (ROS) (Barros *et al.*, 2010).

Rose hips have long been used in traditional folk medicine, but their multiple health properties have drawn more attention and have been investigated more frequently in recent decades. The British Herbal Pharmacopoeia reports that rose hips can be used for gastritis, diarrhoea, polydipsia, and vitamin C deficiency (Pharmacopoeia, 1983). In both laboratory and pre-clinical trials, rose hips have been found to have anti-inflammatory activity, which has been attributed to high concentrations of galactolipids in the fruit (Christensen, 2009; Kharazmi, 2008). In clinical studies, rose hip powder has been found to reduce the pain of osteoarthritis (Christensen *et al.*, 2008; Rossnagel *et al.*, 2007) and to provide moderate benefits for patients with rheumatoid arthritis and chronic back pain (Willich *et*

al., 2010; Chrubasik *et al.*, 2008). Furthermore, rose hips have been shown to possess hypoglycaemic effects in diabetic rats and to produce a modest lowering of total cholesterol in humans, which supports the traditional use in Turkey of rose hips to treat diabetes (Orhan *et al.*, 2009; Rein *et al.*, 2004). A standardised rose hip (*Rosa canina* L.) powder produced in Denmark has been on the market as an herbal remedy in the Scandinavian area for many years and is also readily available in Australia and New Zealand (Cohen, 2012; Winther, 2008).

Besides interest in the anti-inflammatory activity, studies on the anti-cancer activity of rose hips have increased in recent years. Rose hip extract has been found to decrease the proliferation of human glioblastoma cell lines A-172, U-251 MG, and U-1242 MG, human cervix epithelial cancer cell line HeLa, human breast cancer cell line MCF-7 and human colon cancer cell line HT-29, effects which have mainly been attributed to the polyphenols and the abundant carotenoid constituents (Cagle *et al.*, 2012; Tumbas *et al.*, 2012; Olsson *et al.*, 2004).

1.2 Carotenoids

Carotenoids are one of the dominant phytochemical groups in rose hips. The concentration of carotenoids can reach 1 mg/g dry weight in ripe rose hips, which is two-fold and eight-fold higher than the concentration reported for flavonoids and phenolics in rose hips, respectively. The carotenoid content in rose hips is much higher than that in many other fruits and berries, including cherry, raspberry, blueberry, blackcurrant, plum, and apple (Andersson *et al.*, 2011; Olsson *et al.*, 2004).

1.2.1 Structural properties of carotenoids

Carotenoids, which are also called tetraterpenoids, include all derivatives of tetraterpenes. Carotenoids have a long conjugated double chain formed by eight isoprene units. Natural carotenoids exist either in free form or in esterified form. The free form carotenoids are classified as carotenes (which are purely hydrocarbons and contain no oxygen), for example lycopene, α- and β-carotene, and xanthophylls (which contain oxygen), for example violaxanthin, lutein, and zeaxanthin (Figure 2). Due to the existence of the hydroxyl group, xanthophylls can be esterified with fatty acids to form xanthophyll esters.

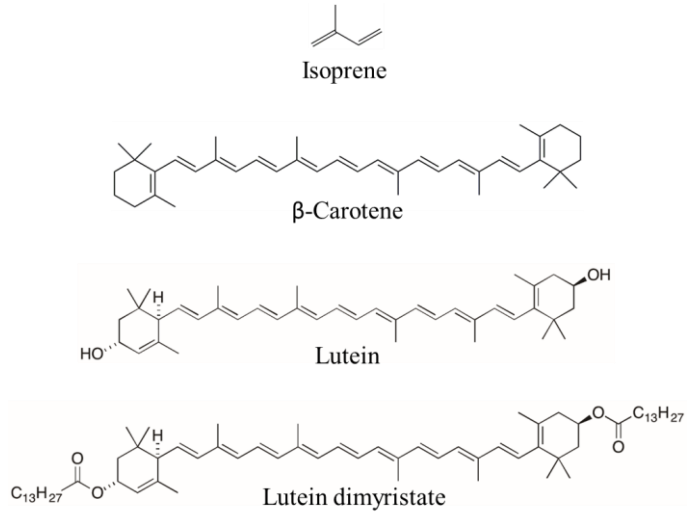

Figure 2. Chemical structure of isoprene, β-carotene (an example of a carotene), lutein (an example of a xanthophyll), and lutein dimyristate (an example of a xanthophyll ester).

The conjugated polyene chromophore determines the light absorption properties, the natural yellow to red colour, the photochemical properties of the molecule, and consequently the photo-protective action in the original organism (Britton, 1995a). Carotenoids have special finger-shaped absorbance spectra in the 400-500 nm wavelength range (Britton, 1995b). Each individual spectrum can be defined by the wavelength of the middle absorption band (λ_{max}) and a numerical notation %III/II, describing the fine structure (Figure 3).

1.2.2 Health effects of carotenoids

Epidemiological studies have shown that increased dietary intake of carotenoids reduces the risk of coronary heart disease in men (Liu *et al.*, 2001) and women (Osganian *et al.*, 2003) and decreases the risk of cardiovascular disease mortality (Gaziano *et al.*, 1995). In elderly men, total plasma carotenoid level can be a health indicator due to its negative association with mortality risk (Akbaraly *et al.*, 2009).

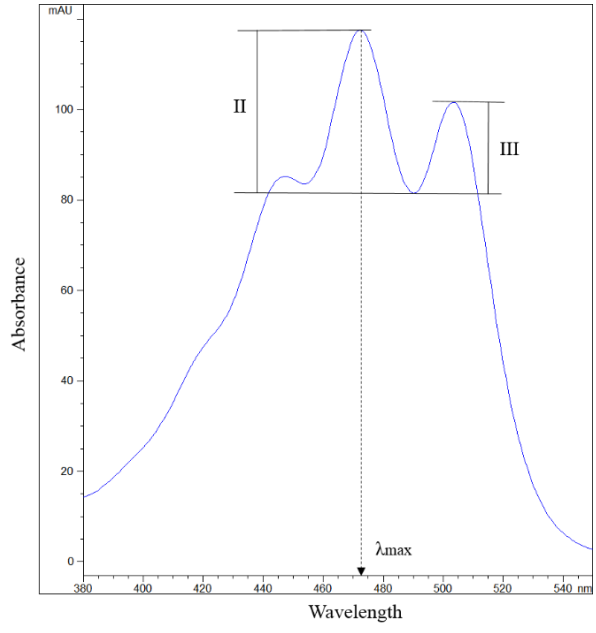

Figure 3. A representative spectral fine structure of lycopene. %III/II is calculated for each individual carotenoid (Britton, 1995b).

The relationship between carotenoids and different types of cancers has also been widely investigated. A positive correlation between plasma carotenoid concentration and recurrence-free survival rate in women diagnosed with early-stage breast cancer has been suggested (Rock *et al.*, 2005). In a large case-control study, lutein, but not other carotenoids, has been found to be inversely associated with colon cancer in both men and women (1993 cases, 2410 controls) (Slattery *et al.*, 2000). Moreover, plasma carotenoid level has been found to be inversely associated with the risk of cervical neoplasia (Potischman & Brinton, 1996) and β-carotene has been found to induce apoptosis in cervical dysplastic cells and prevent cervical carcinogenesis in *in vitro* studies (Muto *et al.*, 1995). No association has been found between lung cancer risk and dietary lycopene or β-cryptoxanthin intake, whereas dose-dependent inverse associations have been found for dietary β-carotene, α-carotene, and lutein. No other carotenoid, but high intake of lycopene, has been found to be associated with a 35% lower risk of total prostate cancer and a 53% lower risk of advanced prostate cancer (Giovannucci, 2002). All these studies indicate the specificity of the anti-cancer properties of different carotenoids in multiple cancer types. Therefore, consuming fruit and vegetables containing various carotenoids, compared with only foods rich in a particular carotenoid, is suggested for a healthy life.

1.3 Triterpenes

Triterpenes are constructed of six isoprene units, which can build nearly 200 different skeletons (Xu *et al.*, 2004). Triterpenes are one of the most diverse groups of plant natural products, with more than 20,000 compounds reported to date. Triterpenes are components of surface waxes, which accumulate in the epicuticular and intracuticular wax layers of stem and leaf surfaces (Buschhaus *et al.*, 2007).

Triterpenes can be classified into different groups according to their biosynthetic pathways. These include the squalene, lanostane, dammarance, lupane, oleanane, ursane, and hopane groups, and a miscellaneous group (Hill & Connolly, 2012). Ursolic acid (UA) (belonging to the ursane group), oleanolic acid (OA) (belonging to the oleanane group), and betulinic acid (BA) (belonging to the lupane group) are representative pentacyclic triterpenes (Hill & Connolly, 2012). Their chemical structures are shown in Appendix 1 in Paper IV.

Ursolic acid concentration has been found to range from 60 to 100 mg per 100 g of fresh matter in cranberry fruit (Kondo, 2006) and UA has also been isolated from blueberry fruit (Wang *et al.*, 2000). Oleanolic acid is the most abundant constituent of grape wax (Radler & Horn, 1965). Both UA and OA are present in sweet cherry fruit wax and in apple peel (Lv *et al.*, 2015; Peschel *et al.*, 2007). A considerable amount of BA is found in the outer bark of a variety of tree species, especially the birch tree (Yogeeswari & Sriram, 2005). These three triterpenes have all been detected in rose hips, with UA constituting 80% of the total triterpene content in *Rosa canina* L. (Wenzig *et al.*, 2008), while OA, UA, and BA are approximately equally distributed in *Rosa multiflora* Thunb. (Paper IV).

Triterpenes have a wide range of applications in the food, cosmetics and pharmaceutical industries (Thimmappa *et al.*, 2014). White birch bark, containing BA, is used by Native Americans as a folk remedy and is used in tea and other beverages to treat stomach and intestinal problems such as diarrhoea and dysentery (Yogeeswari & Sriram, 2005). Both OA and UA, and their derivatives, have well-documented anti-inflammatory (Li *et al.*, 2003), anti-ulcer (Nishino *et al.*, 1988), hepatoprotective (Udayama *et al.*, 1998), anti-diabetic (Ortiz-Andrade *et al.*, 2007), fungicidal (Becker *et al.*, 2005) and anti-parasitic (Cunha *et al.*, 2003) effects. Anti-cancer activity of OA, UA, and BA has been reported for a wide variety of cancer cell lines (Shyu *et al.*, 2010; Srivastava *et al.*, 2010; Petronelli *et al.*, 2009). It has also been found that inhibition of growth and metastasis of human colorectal cancer cell lines by UA can be enhanced in combination with capcetabine (Prasad *et al.*, 2012). A triterpene extract (containing OA, UA, and BA) from Japanese apricot has

been reported to show synergistic cytotoxicity in oesophageal squamous carcinoma cells (YES-2 cells) when combined with the anticancer drug 5-fluorouracil, causing apoptosis and inhibiting metastasis in a mouse model (Yamai *et al.*, 2009). These studies reveal potential to enhance anti-cancer activity by combining triterpenes with other anti-cancer compounds.

1.4 Ascorbate (vitamin C)

Ascorbate/ascorbic acid/vitamin C is the most abundant antioxidant in plant tissues (Walker *et al.*, 2006). It is an important nutrient for normal metabolic functions in humans. Deficiency of ascorbate can lead to various symptoms of scurvy (Padh, 1991). Ascorbate plays an important protective role in the body against damage by free radicals through its free radical scavenging ability and contributes to protection of cellular membranes against lipid peroxidation and damage to DNA and cellular organelles (Bendich *et al.*, 1986). Rose hips are an ascorbate-rich resource. The ascorbate content in rose hips is much higher than many other fruits and berries (Olsson *et al.*, 2004), *e.g.* it can be up to 47.5 mg/g in the richest samples from the species *Rosa cinnamomea* L. and *Rosa. acicularis* Lindl. (Pyke & Melville, 1942).

Besides the well-known antioxidant properties, high doses of ascorbate exhibit pro-oxidant properties, through interaction with metal ions, catalysing their oxidation with concomitant formation of H_2O_2 (Chen *et al.*, 2008; Fisher & Naughton, 2004). Cancer cells are more susceptible to increased oxidative stress by ATP depletion than normal cells. Primary ATP generation in normal cells occurs through oxidative phosphorylation, but in cancer cells glycolysis may play a major role (the Warburg effect) (López-Lázaro, 2008). It has been suggested that H_2O_2 decreases glycolysis in cancer cells and that the mitochondria are more sensitive to H_2O_2, resulting in decreased ATP production. Cancer cells that are specifically glycolysis-dependent could be particularly sensitive to H_2O_2 compared with normal cells (Chen *et al.*, 2007). Therefore, high doses of ascorbate have been used in pro-oxidative therapeutic strategies in cancer treatment (Venturelli *et al.*, 2015).

In humans taking ascorbate orally, the resulting plasma concentration rarely exceeds 80 µM, even up to 2000 mg per day is ingested (Levine *et al.*, 1996). In Sweden the recommended daily intake of ascorbate for adult is 75 mg per day (SLV, 2017). In Sweden, therefore the ascorbate concentrations showing cytotoxicity in cancer cells in culture (mM) can only be achieved by intravenous administration to patients (Verrax & Calderon, 2009). Furthermore, parenterally administered ascorbate (≈0.25-0.5 mg/g body weight) has been shown to preferentially generate $Asc^{\bullet -}$ and H_2O_2 in the

extracellular fluid but not in blood, which has been suggested to provide a foundation for pursuing ascorbate treatment in cancer therapy (Chen *et al.*, 2007).

A previous study reported a positive correlation between the level of vitamin C and the anti-proliferative effect of 10 extracts from different fruits and berries in the HT29 human colon cancer cell line and in the MCF-7 human breast cancer cell line (Olsson *et al.*, 2004). Lately, it has been found that ascorbate improves the therapeutic efficacy of gemcitabine in human pancreatic cancer cell lines (Espey *et al.*, 2011) and acts synergistically with sorafenib in killing hepetocellular carcinoma HepG2 cells, but not in primary hepatocytes (Rouleau *et al.*, 2016). This thesis examined the ability of different combinations of carotenoids, ascorbate, and triterpenes to yield synergistic effects in cancer treatment.

1.5 Human breast cancer and cell lines

Breast cancer originates from breast tissue. Some breast cancers start in the ducts where milk is transported to the nipple (ductal cancers), while other breast cancers have their origin in the glands where the breast milk is generated (lobular cancers) (Sariego *et al.*, 1995). The hormones oestrogen and progesterone promote breast cancer growth and a positive association has been found between increased risk of breast cancer and sex hormone concentrations in both pre-menopausal (Hormones & Group, 2013) (Key *et al*., 2013) and post-menopausal women (Hankinson *et al.*, 1998). Men can also contract breast cancer, but it is about 100-fold more common in women than in men (Hotko, 2013).

Breast cancer is the most frequent carcinoma in women (Lux *et al.*, 2006). Worldwide, 1.7 million new cases were diagnosed in 2012 and the breast cancer death rate is higher than that for any other cancer apart from lung cancer. The chance of a woman dying from breast cancer is about 3% (Torre *et al.*, 2015). Breast cancer is staged from 0 to IV/(4) according to the size of the tumour, whether it has grown into nearby areas, whether it has reached the lymph nodes and whether it has metastasised (American Cancer Society, 2016). The five-year survival rate is 99% for diagnosed early-stage breast cancer patients, but only 23% for advanced-stage patients. The recurrence rate five and ten years after treatment is 11% and 29%, respectively (Brewster *et al.*, 2008).

The heterogeneity of breast cancer has been noted in histological and clinical studies for a long time. Breast cancer is one of the few cancer types

that has so far been subjected to molecular classification for individual therapy, which has significantly improved the clinical outcome (Polyak, 2011).

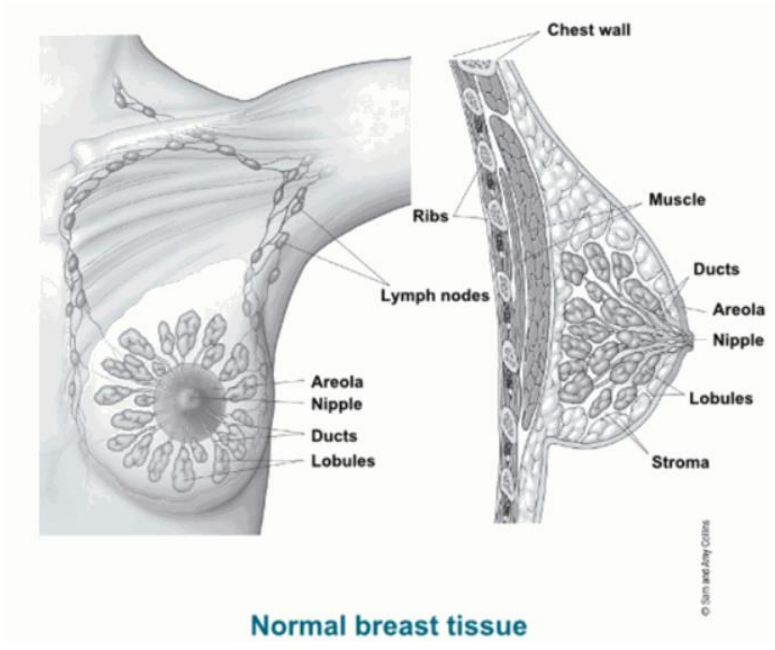

Figure 4. Schematic representation of normal breast tissue (American Cancer Society, 2016).

1.5.1 Breast-derived cell lines

Breast cancers can be classified into different sub-groups according to receptors that exist in the cell membrane, cytoplasm, and nucleus, which bind chemical messengers and induce reactions in cells. The most important receptors for breast cancer classification are hormone receptors (including oestrogen and progesterone receptors) and human epidermal growth factor receptor 2 (HER2) (Vuong *et al.*, 2014). Hormone receptor-positive cancers, which are more common in women after the menopause, can be treated with hormonal therapy drugs that decrease oestrogen levels or block oestrogen receptors (Pusztai *et al.*, 2006). Hormone receptor-negative cancers, which are more common in women before the menopause, can be classified into HER2-positive cancer or triple-negative cancer (cells do not have oestrogen and progesterone receptors or HER2 receptor) (American Cancer Society, 2016). Clinically, the prognosis for patients with HER2 tumours is poorer than that for patients with oestrogen receptor-positive tumours and triple-negative cancer is correlated with a worse prognosis and a higher risk of death (Liu *et al.*, 2013).

In the present thesis, the two human breast cancer cell lines MCF-7 and JIMT-1 were chosen for testing due to their representative characteristics for different breast cancer sub-groups. In addition, the human breast-derived MCF-10A cell line was used as a normal-like cell model for comparison.

JIMT-1 (Figure 5A) is a human ductal carcinoma breast cancer cell line established from a pleural metastasis in a 62-year-old female patient in 2004 (Tanner *et al.*, 2004). The JIMT-1 cells lack expression of oestrogen and progesterone receptors and overexpress HER2 mRNA and protein, therefore are classified into the HER2 sub-group of breast cancers. However, JIMT-1 cells are insensitive to HER2-inhibiting antibodies, for instance trastuzumab, pertuzumab, and lapatinib (Köninki *et al.*, 2010; Tanner *et al.*, 2004). The JIMT-1 cell line is phenotypically of epithelial progenitor cell origin, with a doubling time of about 24 hours.

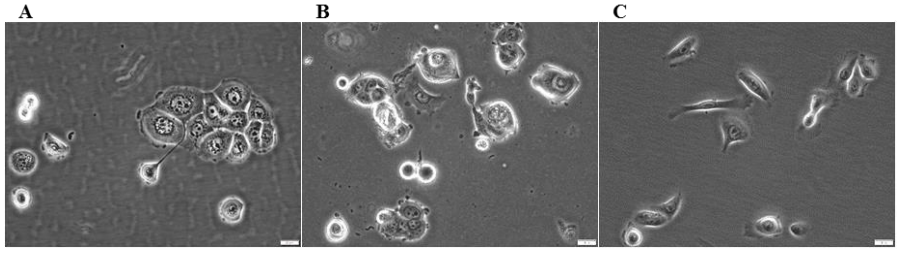

Figure 5. Phase contrast images of the human breast cancer cell lines (A) JIMT-1 and (B) MCF-7, and (C) the human normal-like breast epithelial cell line MCF-10A. Bars = 20 µM. Photos: L. Zhong.

MCF-7 (Figure 5B) is a human breast cancer cell line established from the pleural effusion of a 69-year-old female patient with metastatic mammary carcinoma in 1972 (Soule *et al.*, 1973). MCF-7 is an oestrogen-positive cell line and has proven to be an excellent model for studying the mechanism of tumour response to endocrine therapy and the biological actions of hormones (Levenson & Jordan, 1997; Horwitz *et al.*, 1975). The doubling time of the MCF-7 cell line is about 35 hours.

MCF-10A (Figure 5C) is a normal-like breast epithelial cell line derived from human diploid fibrocystic mammary tissue and has been maintained as an attached immortal cell line since 1990 (Soule *et al.*, 1990). It has been used for studying the mechanism of breast epithelium transformation and in many breast cancer therapy research studies as a normal-like breast epithelial cell model (Ramachandran *et al.*, 2005; Davis *et al.*, 2000; Tannheimer *et al.*, 1998; Ginestra *et al.*, 1997; Soule *et al.*, 1990).

1.5.2 Cancer stem cells

Since the first discovery of leukaemia stem cells about a decade ago (Dick, 1997), attention has been drawn to a cell sub-population now also found in solid tumours that has similar properties to somatic (tissue-specific) stem cells. This cell population has been named tumour-initiating cells or cancer stem cells (CSCs) (Kai *et al.*, 2010). The latter definition is used in this thesis. Like normal tissue-specific stem cells, CSCs possess self-renewal and differentiation abilities, through symmetrical and asymmetrical division, respectively (Figure 6). The microenvironment of the CSCs, which is termed the CSC 'niche', is believed to maintain the properties of CSCs (Reya *et al.*, 2001). During recent years, CSCs have been recognised as important targets for cancer treatments (Dragu *et al.*, 2015).

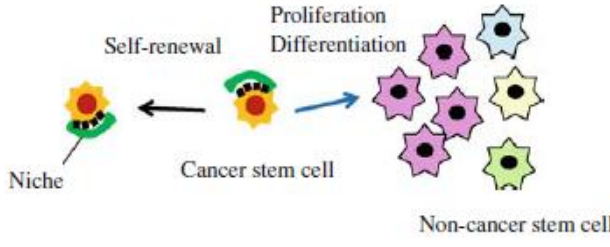

Figure 6. Cancer stem cells (CSCs) and their self-renewal and differentiation abilities, which maintain the hierarchical structure of cancer tissue (Kai *et al.*, 2010).

The suggestion that only a minority of breast cancer cells have the ability to form new tumours was initially put forward by Ai-Hajj *et al.* (2003). Breast cancer cells are heterogeneous with respect to expression of various types of cell surface markers. The same investigation done by Ai-Hajj *et al.* found that the tumour-forming sub-population or the CSC sub-population had increased expression of CD44 and reduced or absent expression of CD24 on the cell membrane ($CD44^{+}/CD24^{-/low}$). This sub-population was found to be highly tumorigenic compared with other phenotypes (Al-Hajj *et al.*, 2003). Later, aldehyde dehydrogenase (ALDH), an enzyme family that catalyses dehydrogenation of aldehydes, was found to be expressed in a sub-population of human normal breast cells and this sub-population was found to have phenotypic and functional characteristics of mammary stem cells (Ginestier *et al.*, 2007). Furthermore, the $ALDH^{+}$ population has been shown to contain a number of up-regulated stem cell-related genes. The presence of $ALDH^{+}$ has also been shown to be a good marker for CSC sub-population identification (Charafe-Jauffret *et al.*, 2009).

Despite the fact that diagnostics and therapeutic treatment for cancer have achieved significant improvements, metastasis still results in about 90% of cancer deaths and is the major cause of cancer mortality (Christofori, 2006; Hanahan & Weinberg, 2000). Clinically, therapeutic agents that inhibit tumour metastasis are highly desirable. Metastasis is a multistep process through which a primary tumour moves from its initial site to secondary tissues or organs (Fidler, 2003; Weiss, 1999). In 2007, it was suggested that the CSCs might be primarily responsible for tumour metastasis (Dalerba *et al.*, 2007). The relationship between CSCs and tumour metastasis has been investigated in pancreatic cancer and it has been shown that the CSC sub-population is essential for this process (Dalerba & Clarke, 2007; Hermann *et al.*, 2007). One of the essential steps in tumour metastasis is cell migration and invasion, through which tumour cells can enter the lymphatic system and blood vessels and thereby spread into secondary organs (Steeg, 2006; Mareel & Leroy, 2003).

Some studies have shown that overexpression of HER2 receptors in a series of breast cancer cell lines increases the $ALDH^+$ CSC sub-population, with increased expression of stem cell regulatory genes, increased invasion and increased tumorigenesis in mice (Korkaya *et al.*, 2008). HER2-positive cell lines are specially enriched in a distinct CSC sub-population defined by $ALDH^+$ (Lesniak *et al.*, 2013). The JIMT-1 cell line, the first commercially established HER2-positive cell line, contains a rather large proportion of cells with ALDH activity and it is used as a model for CSC studies in cell culture studies.

1.5.3 Nuclear factor-κB pathway

Many therapeutic agents exert their activity against cancer cells by affecting various complex signal transduction pathways. The nuclear factor-κB (NF-κB) pathway was initially identified as a regulator in B lymphocytes nearly 30 years ago and has been examined extensively in cancer therapy studies. NF-κB is a family of proteins consisting of five members, p50, p52, p65 (RelA), RelB, and c-Rel, forming homo- and heterodimers, which are responsible for activating transcription of target genes for immunoregulation, inflammation, proliferation, and cell differentiation (Yamamoto *et al.*, 2013; Hayden & Ghosh, 2008). When not active, NF-κB is sequestered in the cytoplasm by association with inhibitory proteins. When triggered by stimuli, NF-κB dimers are released and translocated to the nucleus, where they bind to specific DNA sequences and promote the transcription of target genes (Hayden & Ghosh, 2008).

In normal cells, NF-κB becomes activated only under appropriate stimuli and afterwards returns to the bound inactive state, while in cancer cells it may be constitutively activated due to dysregulation, resulting in impaired regulation of gene expression (Dolcet *et al.*, 2005). Thus, the NF-κB pathway is a target for therapeutic intervention and thus for drug development.

It has been reported that the NF-κB pathway is important for leukaemia stem cells (Guzman *et al.*, 2005; Malaguarnera *et al.*, 2003; Guzman *et al.*, 2001) and that the CSC sub-population of breast cancers can be selectively inhibited by targeting the NF-κB pathway (Yamamoto *et al.*, 2013; Zhou *et al.*, 2008). In this thesis, the NF-κB pathway was investigated as a mechanism of action of some compounds that showed inhibition of the CSC sub-population.

2 Aims and objectives

The overall aims of this thesis were to: 1) determine the carotenoid composition of rose hips from different species; 2) investigate the anti-proliferative activities of different carotenoid fractions and single carotenoid compounds; 3) explore the effects of selected carotenoid fractions and single carotenoids on breast cancer cells and on the CSC sub-population and elucidate the mechanism; and 4) investigate the combined effect of different phytochemicals on cancer cell proliferation.

Specific objectives were to:

- Develop a simple, rapid, and efficient HPLC-DAD-APCI$^+$-MS method for identifying and quantifying the carotenoid composition in rose hips from different rose species (Paper I)

- Investigate the anti-proliferative activity of various carotenoid fractions in rose hips from different rose species in human breast cancer cell lines (Paper II)

- Compare the anti-proliferative activity of xanthophylls and xanthophyll esters on human breast cancer cell lines (Paper III)

- Investigate the inhibitory effect of the most effective carotenoid ester fraction and individual xanthophyll esters on the CSC sub-population and elucidate the mechanism (Papers II and III)

- Investigate the combined anti-proliferative effect of carotenoids, triterpenes and ascorbate in cancer cells and normal-like cells (Paper IV).

The hypotheses tested were that:

- Carotenoid composition and concentration vary between rose species (Paper I)

- Anti-proliferative activity varies between carotenoid fractions, rose species, and cancer cell lines (Paper II)

- Xanthophyll esters are more potent inhibitors of cell proliferation than xanthophylls (Paper III)

- The carotenoid ester fraction from rose hips and individual xanthophyll esters inhibit the CSC sub-population, partly through the NF-κB pathway (Papers II and III)

- The combined effect of carotenoids, triterpenes, and ascorbate on cell proliferation varies between different combinations of compounds, concentrations, and different cell lines (Paper IV)

3 Materials and methods

The procedures used in the analyses are described briefly below. Detailed information on all methods can be found in Papers I-IV.

3.1 Plant materials

Five rose species were used in this study: *Rosa rubiginosa* L. (Figure 7A), *Rosa carolina* L. (Figure 7B), *Rosa multiflora* Thunb. (Figure 7C), *Rosa virginiana* P. Mill (Figure 7D), and *Rosa rugosa* Thunb. (Figure 7E). Rose hips from plants of these five species growing in Alnarpsparken (55°39'N, 13°04'E), Swedish University of Agricultural Sciences, Alnarp, Sweden, were used in the experiments. Fruit harvesting was carried out at the fully ripe stage in the late harvest season, defined according to a previous study (Andersson *et al.*, 2011), in order to obtain high carotenoid levels. Rose hips were picked from two to three bushes for each species and pooled (Figure 7). All samples were stored at -80 °C immediately after harvest until extraction.

Rose hips from *R. rubiginosa*, *R. multiflora*, *R. virginiana*, and *R. rugosa* harvested in autumn 2014 were used in Paper I. Rose hips from *R. rubiginosa*, *R. carolina*, *R. multiflora*, *R. virginiana*, and *R. rugosa* harvested in autumn 2013 were used in Paper II and rose hips from *R. multiflora* harvested in autumn 2014 were used in Paper IV.

Marigold (*Tagetes erecta* L.) flowers were grown in the greenhouse at the Swedish University of Agricultural Sciences, Alnarp, Sweden. Fresh flower petals were harvested and used for extraction of lutein dimyristate (Paper III) and lutein myristate palmitate (Paper II).

Figure 7. Photos showing how the size and colour of rose hips differ between species: (A) *R. rubiginosa*, (B) *R. carolina*, (C) *R. multiflora*, (D) *R. virginiana*, and (E) *R. rugosa*. Bar at right bottom corner of each picture = 1 cm. Photos: L. Zhong.

3.1.1 Dry sample extraction

In general, plant materials for analysis were first freeze-dried, milled, and homogenised. A fixed amount of rose hip powder or marigold petal powder was extracted with an adequate amount of ethanol/n-hexane (v/v, 4/3), hexane, or ethanol depending on the objective (Table 1). After 30 min ultrasonication and 20 h shaking at 4 °C in darkness, the extract was centrifuged at 3000 *g* at 4 °C for 10 min. When carotenoid fractions were isolated, around 1 mL supernatant was saved for quantification. The other part was dried under N_2 and dissolved in tetrahydrofuran (THF) for carotenoid fraction isolation and in ethanol for triterpene fraction (TF) isolation.

3.1.2 Fresh sample extraction

Fresh marigold petals were used for extraction in Paper II. A 30 g portion of fresh petals was homogenised in 200 mL methanol/ethyl acetate/petroleum ether (v/v/v, 1/1/1). After 1 h shaking, the upper phase was collected. The extraction was conducted three times in total. About 20 mg of anhydrous sodium sulphate (Na_2SO_4) were added to the extract to absorb the remaining water. After centrifugation, the supernatant was dried under N_2 and dissolved in 100 μL THF for isolation.

Table 1. *Extraction methods used for different purposes in Papers I-IV*

Purpose	Plant material	Solvent	Paper
Carotenoid identification and quantification	0.5 g rose hip powder	10 mL ethanol/n-hexane (v/v, 4/3)	I
Carotenoid fraction isolation	1.5 g rose hip powder	15 mL ethanol/n-hexane (v/v, 4/3)	II
Lutein ester isolation	1.5 g marigold petal powder	30 mL hexane	III, IV
Triterpene quantification and fraction isolation	5 g rose hip powder	50 mL ethanol	IV

3.1.3 Saponification

In Paper I, the samples were subjected to saponification to determine the xanthophyll composition of the rose hips. In brief, 500 µL of 10% (w/v) KOH in methanol:water (4:1, v/v) solution and 200 mg butylated hydroxytoluene were added slowly to 5 mL carotenoid extract under N_2. After 45 min at 75 °C, 5 mL of 2% (w/v) KCl and 5 mL n-hexane/ethyl acetate (9:1, v/v) were added. The sample was then centrifuged at 3000 *g* for 5 min to obtain the upper organic layer. Extraction after saponification was conducted three times in total. The supernatant was dried under N_2 and dissolved in 5 mL acetone.

3.2 Identification, quantification and isolation of carotenoid fractions/compounds

3.2.1 Identification and quantification of carotenoids in rose hips and procedure for isolation

A high-performance liquid chromatograph equipped with a diode array detector and an atmospheric pressure chemical ionisation (positive mode) mass spectrometer (HPLC-DAD-APCI$^+$-MS) was used for carotenoid identification and quantification in Paper I. HPLC-DAD was used for carotenoid quantification and fraction isolation in Paper II.

A Phenomenex Luna column (3 µ, 100 × 3 mm C18 (2)) with a C18 security guard column was used for identification and quantification and a Phenomenex Luna column (5 µ, 150 × 4.60 mm C18) with a C18 security guard column was used for isolation of fractions. Different elution gradients consisting of solvent A [80% acetonitrile, 15% methanol, and 5% dichloromethane (v/v/v)] and solvent B [30% acetonitrile, 20% methanol, and 50% dichloromethane (v/v/v)] were used according to the purpose of the

analysis. Detection of carotenoids was always carried out at a wavelength of 458 nm.

In Paper I, each carotenoid compound was identified through a combination of absorption spectrum and MS signals. In addition, the fatty acid composition was analysed by gas chromatography (GC). In Paper II, carotenoid fractions were identified and the lycopene isomer fraction, undefined carotene fraction and xanthophyll ester fraction were isolated mainly according to their elution sequence. All carotenoid compounds or fractions were quantified with β-carotene as external standard.

3.2.2 Isolation of lutein esters from marigold petals

Lutein dimyristate and lutein myristate palmitate isolation was carried out with a semi-preparative Phenomenex Luna column (10 µ, 250 × 10.00 mm C18) with a C18 security guard column. The eluent consisted of 50% acetonitrile, 18% MeOH and 32% dichlormethane (v/v/v). Flow rate was 5 mL/min for 80 min. Lutein dimyristate was isolated between the 27th and 31st min (Figure 2A in Paper III) and lutein myristate palmitate was isolated between the 34th and 38th min (Figure S1 in Paper II).

3.3 Quantification of triterpenes and isolation of the triterpene fraction

Separation and subsequent quantification of triterpenes in rose hips were conducted using an YMC Triart C18 ExRS plus column (3 µm, 150 x 3.0 mm, 8 nm). Triterpene fraction isolation was carried out with a VYDAC column. Compounds were eluted using 100% acetonitrile and detected at 207 nm wavelength. External standards of UA, OA, and BA were used for quantification (Paper IV).

3.4 Chemical synthesis of zeaxanthin dimyristate

The synthesis procedure was based on a previous study (Khachik & Beecher, 1988). Zeaxanthin dimyristate was produced by esterification of zeaxanthin and myristoyl chloride under N_2 as atmosphere in limited light. The target product was isolated and purified using solid phase chromatography.

Both lutein dimyristate and zeaxanthin dimyristate were quantified using HPLC, with an Agilent Eclipse Plus C18 column (3.5 µm, 3.0 x 100 mm). The eluent consisted of 40% acetonitrile, 19% methanol, and 41% dichloromethane (v/v/v) with 1 mL/min flow rate for 5 min. β-Carotene was used as external standard (Paper III).

3.5 Cell culture conditions

The two human breast cancer cell lines JIMT-1 and MCF-7 were used in Paper II and the human breast epithelial normal-like cell line MCF-10A was used in addition in Papers III and IV. All cell lines were cultured in their individual formulation of medium. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The JIMT-1 and MCF-10A cells were routine-passaged twice a week and the MCF-7 cells once a week.

When adding compounds, carotenoids and triterpenes were always first dissolved and diluted to a stock in phosphate-buffered saline (PBS) with 0.1% Tween 80. When compounds or fractions were added to the cell culture medium to the desired concentration, the final concentration of Tween 80 in the cell culture medium was no higher than 0.01%, a non-toxic concentration for cells (Figure S2 in Paper II). In Paper IV, a stock ascorbate solution was made fresh in PBS every time just before use.

3.6 MTT assay

A tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used in this thesis. The MTT assay has been widely adopted as a 96-well format cell proliferation indicator in thousands of published articles, based on the simplicity of the protocol. Cells of different cell lines were seeded in 96-well plates in 180 µL medium and incubated for 24 h, and then 20 µL of compound in different concentrations were added to the cells (Figure 8A). After 72 h of incubation, a MTT solution was added to the cells.

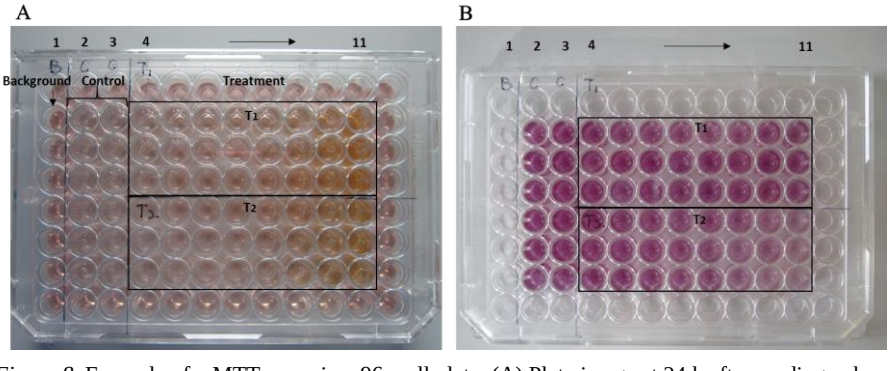

Figure 8. Example of a MTT assay in a 96-well plate. (A) Plate image at 24 h after seeding, when compound was added at increasing concentrations from row 4 to row 11. (B) MTT was reduced to formazan by the living cells and the formazan crystals were dissolved in dimethyl sulphoxide (DMSO), producing a purple solution. The letter B written on the plates means background (row 1), C means control (rows 2 and 3), and T_1 and T_2 mean different treatments (rows 4 to 11). Photos: L. Zhong.

Living cells reduce MTT to purple formazan (Mosmann, 1983) in the mitochondrial electron transport chain and the absorbance of the purple formazan at 540 nm reflects the number of living cells (Figure 8B). The formazan crystals were dissolved in dimethyl sulphoxide (DMSO) and the absorbance of the solution was monitored in a spectrophotometer. Dose response curves were drawn and concentration giving 50% inhibition (IC_{50}) was obtained using the Graphpad software (Papers II, III, and IV).

3.7 Cancer stem cell population $ALDH^+$ assay

JIMT-1 cells were seeded in Petri dishes and incubated for 24 h. Compounds were added and cells were then incubated for 72 h. The cells were detached with Accutase™ and collected in PBS containing 1% foetal calf serum (FCS). The cell density was determined by counting in a haemocytometer and a ALDEFLUOR assay kit was used to determine the number of $ALDH^+$ cells according to the protocol in a previous study (Huang *et al.*, 2016) (Papers II and III).

3.8 Cell migration assay

JIMT-1 cells were seeded to achieve high density in Petri dishes. After 24 h incubation, the medium was removed and three parallel wounds were made in the cell layer by scratching. To minimise wound healing resulting from cell proliferation, serum-free medium was added and then the compounds were added before continued incubation. The wound area was photographed using a 10x objective in an inverted phase contrast microscope at 0, 24, and 48 h of incubation. The wound area was measured using Image J software and wound closure was calculated (Papers II and III).

3.9 Nuclear factor-κB translocation assay

JIMT-1 cells were seeded in 12-well plates containing a round glass cover slip in each well. The medium was replaced with medium containing 0.1% FCS after 48 h of incubation. Compounds were added and incubation continued for 1 h, after which tumour necrosis factor-α (TNF-α) was added and incubation continued for 40 min. The cells were then fixed with 3.7% formaldehyde. Blocking buffer was used to block nonspecific binding sites and to permeabilise the cells. Rabbit anti-p65/NF-κB primary antibody and Alexa 488 anti-rabbit-conjugated secondary antibody were then added sequentially to the samples, with the incubation time with each antibody being 1 h. Thereafter, the

cover slips were mounted in Mowiol on slides and the cells were observed using a 100x objective in an epifluorescence microscope. Images were taken and the number of NF-κB positive cells was determined by manual counting in the images (Papers II and III).

4 Results and discussion

4.1 HPLC-DAD-APCI$^+$-MS method development and carotenoid identification

One of the aims of this thesis was to develop an adequate HPLC-DAD-APCI$^+$-MS method for the identification and quantification of carotenoids in rose hips. A simple, rapid method was established on the basis of a previous HPLC-DAD method (Andersson *et al.*, 2011) on a short 100 x 3 mm reverse phase C18 column with 0.8 mL/min gradient elution for 22 min for each sample. An APCI was fitted in the MS detector and positive mode was chosen to identify xanthophyll esters. In addition, the total lipid composition of rose hips was determined in the process, to investigate the fatty acid part of the esterified xanthophylls using GC (Table 2 in Paper I).

The changes in the chromatograms of carotenoids before and after saponification reflected the xanthophylls that existed in ester form in rose hips (Figure 1 in Paper I). Xanthophyll and carotene identification was based on the elution sequence, retention time of the peaks and individual absorbance spectra parameters, including λ_{max}, %III/II (Britton, 1995a; Britton, 1995b) and $\%A_B/A_{II}$ (the ratio of the cis peak absorbance (A_B) to the middle peak absorbance (A_{II})) (Goupy *et al.*, 2013; Britton, 1995b). In MS analysis, xanthophylls usually lose one or two H_2O molecules, resulting in $[M+H-18]^+$ or $[M+H-18-18]^+$ fragments, respectively. The abundance of the MS signal fragments also provides information on molecular structure difference, for instance lutein and zeaxanthin isomers (Delgado-Pelayo & Hornero-Méndez, 2012) (Table 1 in Paper I).

Identification of xanthophyll esters was based on the combination of absorption spectrum obtained from the DAD detector and the protonated molecular mass $[M+H]^+$ and molecular fragments obtained from the MS detector. Xanthophyll esters losing one molecule of capric acid (C10:0), lauric

acid (C12:0), myristic acid (C14:0) or palmitic acid (C16:0) resulted in $[M+H-172]^+$, $[M+H-200]^+$, $[M+H-228]^+$ or $[M+H-256]^+$ fragments, respectively, in MS signalling (Table 3 in Paper I).

Eleven xanthophylls and 10 carotenes were detected in the saponified extract from rose hips and 23 xanthophyll esters were detected in the unsaponified extract from rose hips.

4.2 Carotenoid content in rose hips

To better describe and compare the carotenoid content in rose hips, in this thesis the carotenoids were divided into five groups: xanthophylls, lycopene isomers, undefined carotenes, β-carotene, and xanthophyll esters (Figure 1 in Paper II). As shown in Figure 9, lycopene isomers, undefined carotenes, and xanthophyll esters were the dominant carotenoids in rose hips. The β-carotene concentration was relatively stable between species and years. Around 80-90% of the xanthophylls were esterified and were present as xanthophyll esters in rose hips harvested during the late season in this thesis.

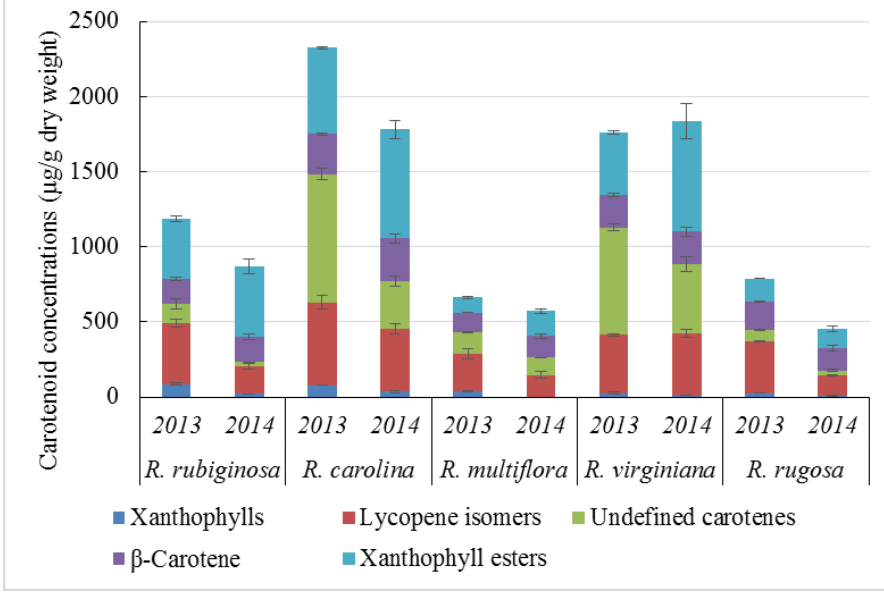

Figure 9. Carotenoid concentration (µg/g dry weight) in rose hips from five rose species collected during 2013 and 2014.

The carotenoid content in rose hips differed between species and years. The total carotenoid content in rose hips from *R. carolina* and *R. virginiana* reached 1.7 mg/g dry weight, which was much higher than that in rose hips from *R.*

rubiginosa, *R. multiflora*, and *R. rugosa*. In general, fruits from 2013 contained slightly higher amounts of carotenoid than fruits from 2014 except for *R. virginiana*. This difference was assumed to reflect the different climate conditions between these two years, where the summer temperature in 2014 was somewhat warmer than in 2013 and the total number of sunshine hours in June, July, and August 2014 was greater than in 2013 (SMHI, 2013 & 2014). The rose hips were harvested based on their colour and ripening time was approximately one week earlier in 2014 than in 2013. The total carotenoid content was lower in 2014 than in 2013, which was correlated to a decrease in either the lycopene fraction or the undefined carotene fraction (Figure 9). Similarly, Andersson *et al*. (2011) demonstrated that the content of lycopene and total carotenoids in rose hips has a significant negative correlation with temperature and irradiation, which confirms the influence of climate on the variation in carotenoid content in rose hips between years. Interestingly, even though the total carotenoid content in rose hips in 2014 was generally lower than in 2013, the xanthophyll ester content was increased for four rose species out of five. These differences could be related to the carotenoid synthesis mechanism in plants as a reaction to external environmental stimuli or stress.

4.3 Anti-proliferative effects of carotenoids, triterpenes, and ascorbate

4.3.1 Anti-proliferative effects of different carotenoid fractions from rose hips

In Paper II, the anti-proliferative effects of the total carotenoid extract (TCE) and different carotenoid fractions from rose hips, including the lycopene isomer fraction, undefined carotene fraction, and xanthophyll ester fraction, were investigated in the two human breast cancer cell lines JIMT-1 and MCF-7. The IC_{50} was used as a parameter for evaluation and comparison of anti-proliferative activity.

As shown in Figures 2 and 3 in Paper II, the xanthophyll ester fraction showed similar anti-proliferative activity to TCE and was obviously more potent than the lycopene isomer and undefined carotene fractions, as evidenced by single-digit μg/mL IC_{50} values in most cases (Table 2 in Paper II). Both TCE and the xanthophyll ester fraction from *R. multiflora* hips showed higher anti-proliferative activity than those from the other species. Interestingly, the xanthophyll ester composition in rose hips from *R. multiflora* was found to be different from that in hips from the other species. As reported in Paper I, the dominant peaks of the xanthophyll ester fraction in *R. multiflora* were lutein dilaurate (C12:0-C12:0), zeaxanthin dilaurate (C12:0-C12:0), and a relatively high proportion of violaxanthin dilaurate (C12:0-C12:0), while in the other

species the dominant peaks were rubixanthin laurate (C12:0) and rubixanthin myristate (C14:0).

Anti-cancer activity of other terpenoid esters, but not of xanthophyll esters, has been reported. A sesquiterpene ester extracted from the root of *Celastrus orbiculatus* has been found to reverse the multi-drug resistance property of MCF-7/ADR cells and human oral epidermal cancer KB-V1 cells (Kim *et al.*, 1998). Five daphnane diterpene esters extracted from *Daphne genkwa* (Thymelaeaceae) flowers have been found to inhibit the proliferation of A549 human lung cancer cells, but to be relatively non-cytotoxic in normal lung epithelial MRC-5 cells (Hong *et al.*, 2010). Isolated UA hydroxycinnamate esters can inhibit the proliferation of several lung, colon, breast, and renal cancer cell lines, as well as melanoma and leukaemia cell lines with 1.2 to 11 μM IC_{50} values (Kondo, 2006). To investigate the anti-proliferative activity of xanthophyll esters, MTT assays were used in Papers II and III. The anti-proliferative activity of the free xanthophylls lutein and zeaxanthin and their respective esters were compared in Paper III.

In Paper III, neither lutein nor zeaxanthin showed any inhibitory effect in cell proliferation up to a concentration of 100 μM (Figure 3A in Paper III). Lutein and zeaxanthin have been reported to inhibit human oral epithelial cancer KB cells after 72 h of incubation, with IC_{50} values of 50 μM and 30 μM, respectively (Sun & Yao, 2007). Thus, the same compounds have different effects on different cell types. Both lutein dimyristate (Figure 3B in Paper III) and lutein myristate palmitate (Figure 4 in Paper II) resulted in IC_{50} values in the single-digit μM range and zeaxanthin dimyristate resulted in IC_{50} values in the single- to double-digit μM range (Table 2). It was therefore confirmed that the xanthophyll esters were significantly more potent than their respective free xanthophyll forms.

Table 2. *IC_{50} values (μM) of xanthophylls and xanthophyll esters obtained in MTT assay using the JIMT-1 and MCF-7 human breast cancer cell lines and the MCF-10A normal-like human breast epithelial cell line*

Compound	**JIMT-1**	**MCF-7**	**MCF-10A**
Lutein	> 100	> 100	> 100
Zeaxanthin	> 100	> 100	> 100
Lu-C14-C14[1]	2.25 ± 0.40	0.85 ± 0.16	2.32 ± 0.48
Lu-C14-C16[2]	6.34 ± 0.14	2.19 ± 0.48	NI[4]
Zea-C14-C14[3]	~ 50	6.80 ± 0.95	18.28 ± 6.30

[1] Lutein dimyristate.
[2] Lutein myristate palmitate.
[3] Zeaxanthin dimyristate.
[4] Not investigated.

In addition, the cell proliferation data showed that lutein esters were more potent than zeaxanthin esters and that lutein dimyristate was more potent than lutein myristate palmitate. This suggests that the xanthophyll component, as well as the fatty acid component, in the xanthophyll ester structure influence the bioactivity of the compounds. Further investigations are needed to increase understanding of the difference in bioactivity of various xanthophyll esters.

Comparison with reported IC_{50} values for conventional breast cancer chemotherapy drugs such as mitoxantrone (0.34 µM), chlorambucil (3.4 µM), cyclophosphamide (6.6 µM), doxorubicin (9.6 µM), cis-platinum (14 µM), and 5-fluorouracil (28 µM) in MCF-7 cells, and with liposomal-delivered doxorubicin in JIMT-1 cells (59 µM) after 72 h treatment (Tan *et al.*, 2010; Mahoney *et al.*, 2003), indicates that the two lutein diesters investigated have a more potent anti-proliferative activity than all except mitoxantrone. Although the data obtained from cell lines of xanthophyll ester treatments are thus interesting, many more preclinical studies are needed before these compounds can be considered as chemotherapeutic drugs for clinical trials.

Xanthophyll esters are believed to be hydrolysed by hydroxyl ester lipases in the gut before being taken up by the intestinal mucosal cells and esters are not normally found in blood (Zaripheh & Erdman, 2002). For example, Wingerath *et al.* (1995) showed that an increased amount of free β-cryptoxanthin was present in chylomicrons and serum, but no ester, after intake of a β-cryptoxanthin ester-enriched tangerine concentrate. On the other hand, xanthophyll esters have been detected in human skin at a low level (pmol/g human skin) after intake of tangerine juice concentrate (Wingerath *et al.*, 1998). Moreover, lutein esters (3% of the total serum lutein content) have been found in serum after food supplementation with a natural source of lutein mixture (extract from marigold flower) (Granado *et al.*, 1998), suggesting probable re-esterification of xanthophylls following absorption. Furthermore, human CaCo-2 cells have been found to be able to esterify xanthophylls (Sugawara *et al.*, 2009). However, due to the hydrolysis of xanthophyll esters in the gut and the low ratio of re-esterification of xanthophylls in the human body, xanthophyll esters administered orally will presumably not result in serum concentrations that are inhibitory to cancer cells. The clinical chemotherapeutic drugs used nowadays for breast cancer treatment are typically given either through injection or infusion (American Cancer Society, 2016). A functional concentration of xanthophyll esters could possibly be achieved by parenteral administration.

Even though chemotherapy is one of the most efficient treatment modalities applied in clinical oncology, severe side-effects are usually associated with it, resulting in different degrees of damage to tissues and organs (Ratajczak *et al.*,

2013). Thus, chemotherapeutic drugs with a low degree of side-effects are needed. In this context, it must be pointed out that the xanthophyll esters were also toxic to the normal-like MCF-10A cells tested in this thesis. However, it should also be noted that the oestrogen receptor-positive MCF-7 cells were more sensitive to the esters than the other two cell lines. The implications of this observation need further investigation.

4.3.2 Anti-proliferative effects of combinations of lutein dimyristate, triterpenes, and ascorbate

Both triterpenes and ascorbate have been reported to have synergistic anti-cancer effects in many cancer cell lines in combination with other compounds (Rouleau *et al.*, 2016; Prasad *et al.*, 2012; Espey *et al.*, 2011; Yamai *et al.*, 2009; Olsson *et al.*, 2004). Therefore ascorbate and triterpenes (OA, UA, BA, and TF from *R. multiflora* hips) and the xanthophyll ester lutein dimyristate were used in combination in Paper IV.

When the compounds or TF were added to cells singly, the anti-proliferative activity found differed as follows: ascorbate < BA < OA < UA < TF < lutein dimyristate (Table 1 in Paper IV).

Among the combinations of lutein dimyristate and various triterpenes tested, the IC_{50} of lutein dimyristate combined with 10 or 50 µM triterpenes resulted in synergistic effects mostly in the MCF-7 cell line. For some combinations, antagonistic effects were seen in JIMT-1 cells, while the MCF-10A normal-like cells were less sensitive (Figure 1 in Paper IV). A combination of 10 µM TF and 0.025 - 0.1 mM ascorbate (in particular 50 µM ascorbate) was found to be the most promising combination, inhibiting the proliferation of MCF-7 cancer cells without influencing the MCF-10A cells (Figure 2 in Paper IV).

Previous studies have demonstrated that a high oral dose of ascorbate has no benefit in overall survival of cancer patients (Moertel *et al.*, 1985; Creagan *et al.*, 1979) and that the ascorbate concentration inducing cytotoxicity in cancer cells can only be achieved by intravenous administration (Verrax & Calderon, 2009). However, 50 µM ascorbate, which when administered together with 10 µM TF resulted in a synergistic effect in MCF-7 breast cancer cells, could easily be obtained by a daily ascorbate oral intake of 100 mg (Levine *et al.*, 1996). Thus, the data obtained in this thesis suggest that an oral dose of ascorbate could enhance the toxicity of certain therapeutic compounds in cancer treatment.

4.4 Inhibitory effects of carotenoids on cancer stem cell ALDH$^+$ population

ALDH enzyme activity is a CSC sub-population marker in breast cancer (Charafe-Jauffret *et al.*, 2009) and JIMT-1, a HER2-positive cell line, contains a distinct CSC sub-population defined by ALDH$^+$ (Huang *et al.*, 2016; Lesniak *et al.*, 2013). One of the characteristics of CSCs is their innate resistance to chemotherapy-induced cell death, which is called multiple drug resistance (MDR) (Eyler & Rich, 2008). CSCs express high levels of ATP-binding cassette transporters which mediate drug efflux, resulting in increased resistance to conventional chemotherapeutic drugs such as paclitaxel, doxorubicin, 5-fluorouracil, and cisplatin (Dean *et al.*, 2005; Hirschmann-Jax *et al.*, 2004). This MDR property of CSCs allows them to survive and initiate tumour recurrence, impeding efficacious cancer treatment (Donnenberg & Donnenberg, 2005; Kessel *et al.*, 1968). Therefore drugs targeting CSCs are required. In this thesis, the effects of the xanthophyll ester fraction isolated from *R. multiflora* hips and of xanthophyll esters on CSCs were investigated by monitoring the ALDH$^+$ sub-population in the JIMT-1 cell line (Table 3).

In Papers II and III, the ester fraction and all xanthophyll esters studied were found to significantly reduce the CSC sub-population defined by ALDH$^+$ cells compared with the control. Treatments with IC_{50} of the ester fraction or lutein myristate palmitate reduced the CSC population by almost 50% *i.e.* in the same range as the total cell number reduction in the MTT assay.

Table 3. *Effect of treatment with xanthophyll ester fraction or xanthophyll esters in reducing the ALDH$^+$ sub-population in JIMT-1 cells. Values are presented as percentage of control value.*

Compound	IC_{25}	IC_{50}
Ester fraction	85.7 ± 7.7	55.0 ± 19.3
Lu-C14-C14[1]	88.1 ± 8.4	84.6 ± 13.6
Lu-C14-C16[2]	79.8 ± 13.7	53.2 ± 20.5
Zea-C14-C14[3]	64.2 ±14.8	80.5 ± 12.4

[1] Lutein dimyristate.
[2] Lutein myristate palmitate.
[3] Zeaxanthin dimyristate.

4.5 Inhibitory effects of carotenoids on cell migration

Metastasis is the major cause of cancer mortality, resulting in about 90% of cancer deaths (Christofori, 2006; Hanahan & Weinberg, 2000). It has been reported that CSCs are responsible for cancer metastasis (Dalerba & Clarke, 2007; Hermann *et al.*, 2007). Cell migration is one of the most important steps

in cancer metastasis (Steeg, 2006; Mareel & Leroy, 2003). One method using cell lines to investigate effects of compounds on cell migration is the wound healing assay (Zahm *et al.*, 1997). After scratching of the monolayer, the cell-cell contact is disrupted and the cells at the wound margin start healing the wound through proliferation and migration (Zahm *et al.*, 1997; Coomber & Gotlieb, 1990). To limit the effect resulting from cell proliferation, serum-free medium was used in the wound healing assay in this thesis.

Wound closure was decreased to different extents by treatment with the ester fraction isolated from *R. multiflora* hips and the xanthophyll esters at concentration giving 25% inhibition (IC_{25}) or IC_{50} (Figure 10, see also Figure 5A in Paper II and Figure 4A in Paper III), but not by lutein or zeaxanthin treatment at a concentration of 20 μM (data not shown). Compared with the control, treatment with the ester fraction or xanthophyll esters resulted in significant inhibition of cell migration 24 h after scratching (except lutein dimyristate) and 48 h after scratching (except lutein dimyristate and IC_{25} of zeaxanthin dimyristate) (statistics are shown in Figure 5B in Paper II and in Figure 4B in Paper III).

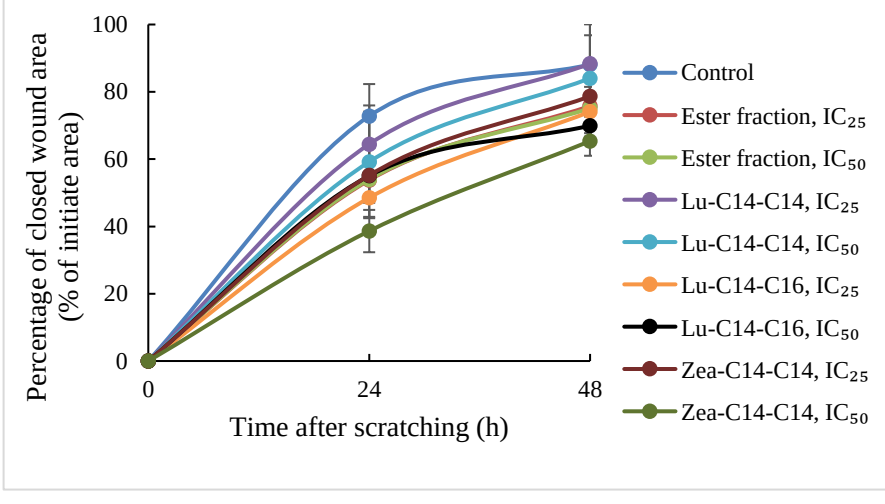

Figure 10. Effects of treatments on wound closure. Treatments: IC_{25} and IC_{50} of the ester fraction isolated from *R. multiflora* hips, lutein dimyristate (Lu-C14-C14), lutein myristate palmitate (Lu-C14-C16), and zeaxanthin dimyristate (Zea-C14-C14).

The xanthophyll esters investigated in this thesis, especially lutein myristate palmitate, significantly inhibited cell migration at the concentrations reducing the number of cells. Interestingly, lutein myristate palmitate treatment reduced the $ALDH^+$ population more efficiently than lutein dimyristate treatment, which might have been reflected in the wound healing assay.

4.6 Inhibition of TNF-α-induced NF-κB translocation to the nucleus by xanthophyll esters

TNF-α is a pleotrophic polypeptide cytokine which plays a significant role primarily in immune activities by activating the transcription factor NF-κB (Feuerstein *et al.*, 1993). When not active, NF-κB is sequestered in the cytoplasm by inhibitory proteins. TNF-α treatment induces phosphorylation of the inhibitory proteins, which results in release of NF-κB. Free NF-κB translocates from the cytoplasm to the nucleus to start regulating the transcription of various proteins important for cell proliferation and inflammatory responses (Beg & Baltimore, 1996; Baeuerle & Henkel, 1994).

In Papers II and III, the effects of the xanthophyll ester fraction isolated from *R. multiflora* hips and of other xanthophyll esters on TNF-α-induced NF-κB translocation to the nucleus were investigated. Representative images are shown in Figure 6A in Paper II and in Figure 6A in Paper III. In this assay, TNF-α treatment alone resulted in 96% of nuclei becoming NF-κB positive. After 1 h of treatment with IC_{50} of the ester fraction or the xanthophyll esters before TNF-α treatment, both lutein dimyristate and the ester fraction slightly reduced the number of NF-κB-positive nuclei. However, lutein myristate palmitate treatment reduced the number of NF-κB-positive nuclei to 35% (Figure 11). On increasing the treatment time of lutein dimyristate to 4 h instead of 1 h before adding TNF-α, the number of NF-κB-positive nuclei decreased to 82% (compared with 90% after 1 h pre-treatment) (Figure 6 in Paper III). Treatment with IC_{50} of zeaxanthin dimyristate for 4 h decreased the number of positive nuclei to 21% (Figure 6 in Paper III). Previous studies have reported that emodin and curcumin inhibit TNF-α-induced NF-κB translocation in a dose-dependent manner (Plummer *et al.*, 1999; Kumar *et al.*, 1998), while the results in this thesis show that time is also important.

Nuclear factor-κB is an important transcription factor involved in CSC maintenance and is a promising target in cancer therapy, especially in targeting the CSC population (Liu *et al.*, 2010; Murohashi *et al.*, 2010; Zhou *et al.*, 2008). The xanthophyll esters studied in this thesis have been proven to partly react in cancer cells through the NF-κB pathway, but this pathway is complex, with regulatory steps on different levels (Gilmore & Herscovitch, 2006) and more detailed studies are required to better understand the targets of xanthophyll esters in this pathway. The findings in this thesis provide a general direction for further mechanistic studies on xanthophyll esters in breast cancer treatment.

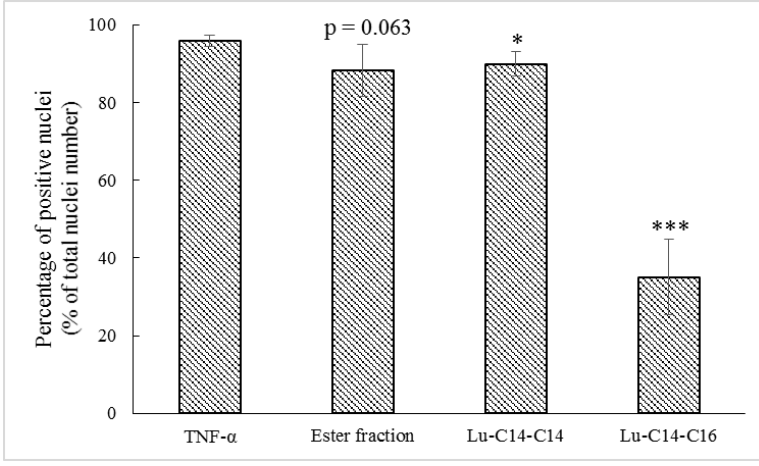

Figure 11. Inhibition of TNF-α-induced NF-κB translocation to the nucleus by 1 h treatment with IC_{50} of ester fraction isolated from *R. multiflora* hips, lutein dimyristate (Lu-C14-C14), and lutein myristate palmitate (Lu-C14-C16).

5 Conclusions and future perspectives

The main conclusions of this thesis are as follows.

- A simple and efficient HPLC-DAD-APCI$^+$-MS method was developed for identification and quantification of carotenoids in rose hips. Twenty-one carotenoids, including 11 xanthophylls and 10 carotenes, were detected in saponified extract and 23 carotenoid esters in unsaponified extract of rose hips from five different rose species.

- The carotenoid content in rose hips differed between species and years. Lycopene, undefined carotene, and xanthophyll esters were dominant carotenoid components in rose hips. The highest carotenoid content, 2.3 mg/g dry weight, was found in *Rosa carolina* L. hips in 2013.

- A lower IC_{50} value in the human breast cancer cell lines JIMT-1 and MCF-7 in MTT assay was found for xanthophyll ester fraction than for lycopene isomer and undefined carotene fractions. The xanthophyll ester fraction from *Rosa multiflora* Thunb. hips was more potent than that in hips from the other species tested, which may be attributable to the unique xanthophyll ester composition of *R. multiflora*, which includes lutein dilaurate, zeaxanthin dilaurate, and violaxanthin dilaurate.

- Xanthophyll esters were more potent in cell proliferation inhibition than their respective free xanthophyll forms, in both cancer cell lines and a normal-like cell line.

- The xanthophyll ester fraction isolated from *Rosa multiflora* Thunb. hips and the xanthophyll esters lutein dimyristate, lutein myristate palmitate, and zeaxanthin dimyristate were found to decrease the $ALDH^+$ CSC sub-population and inhibit cell migration.

- The xanthophyll esters partly exerted their effects through inhibiting the NF-κB pathway.

- Synergistic and antagonistic effects in cell proliferation inhibition were found in MCF-7 and JIMT-1 cell lines, respectively, when lutein dimyristate was combined with triterpenes. The best combination effect was found when an orally viable serum concentration of ascorbate (50 µM) was combined with 10 µM TF isolated from *Rosa multiflora* Thunb. hips. This combination significantly inhibited the MCF-7 cancer cell line without affecting the normal-like MCF-10A breast epithelial cells.

In future investigations on natural compounds in rose hips and xanthophyll esters, there are many openings to pursue. These include:

- Studying the mechanism of carotenoid synthesis in rose hips, especially the esterification procedure, and further exploring the difference between species from a genetic perspective. This could be useful if carotenoid content and composition are included as considerations in future rose hip breeding.

- Testing the bioactivity of other phytochemicals, for example phenolic compounds, in rose hips in order to explore their nutritional role.

- Defining the contribution of OA, UA, and BA to the synergistic effect of combinations of ascorbate and TF and investigating other anti-cancer activities of this combination for possible development as chemotherapeutic agents.

- Investigating and comparing the anti-cancer effect of xanthophyll esters constructed from different xanthophylls (*e.g.* violaxanthin esters) and different fatty acids (*e.g.* lutein dilaurate, zeaxanthin dilaurate), in order to gain a better understanding of molecular

structure and toxicity. The toxicity in normal cells should always be taken into consideration.

- Investigating other cancer types regarding the effect of xanthophyll esters, *e.g.* pancreatic cancer, which is especially treatment-resistant with a dismal outcome.

- Exploring more detailed mechanisms of the NF-κB pathway and investigating the nature of the specific protein or process targeted by the compounds. This would help to further utilise xanthophyll esters in cancer therapy.

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