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DOTTORATO DI RICERCA IN BIOTECNOLOGIE
XXIV CICLO

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New phytopharmaceutical anti-breast cancer formulations: immunoliposomes containing extra virgin olive oil polyphenols

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Introduction

1. The cancer burden

Every year 12 million people worldwide die of the results of atherosclerosis, heart infarctions, and strokes. These are the most common causes of death of our time. The second largest common disease is cancer. Coronary disease and cancer together are responsible for over 80% of all deaths in industrialized countries. In many Third World countries cancer incidence appears much lower, most likely because of the higher death rates due to infectious disease or injury . Based on the most recent incidence and mortality data available, there were 10.1 million new cases, 6.2 million deaths and 22.4 million persons living with cancer in the year 2000. In terms of incidence, the most common cancers worldwide (excluding non-melanoma skin cancers) are lung (12.3% of all cancers), breast (10.4%) and colorectum (9.4%) (**Fig.1**). Lung cancer is the largest single cause of deaths from cancer in the world (1.1 million annually), since it is almost invariably associated with poor prognosis. On the other hand, appropriate intervention is often effective in avoiding a fatal outcome following diagnosis of breast cancer. Hence this particular cancer, which ranks second in terms of incidence, is not among the top three causes of death from cancer, which are respectively cancers of the lung (17.8% of all cancer deaths), stomach (10.4%) and liver (8.8%) (**Fig. 1**) .

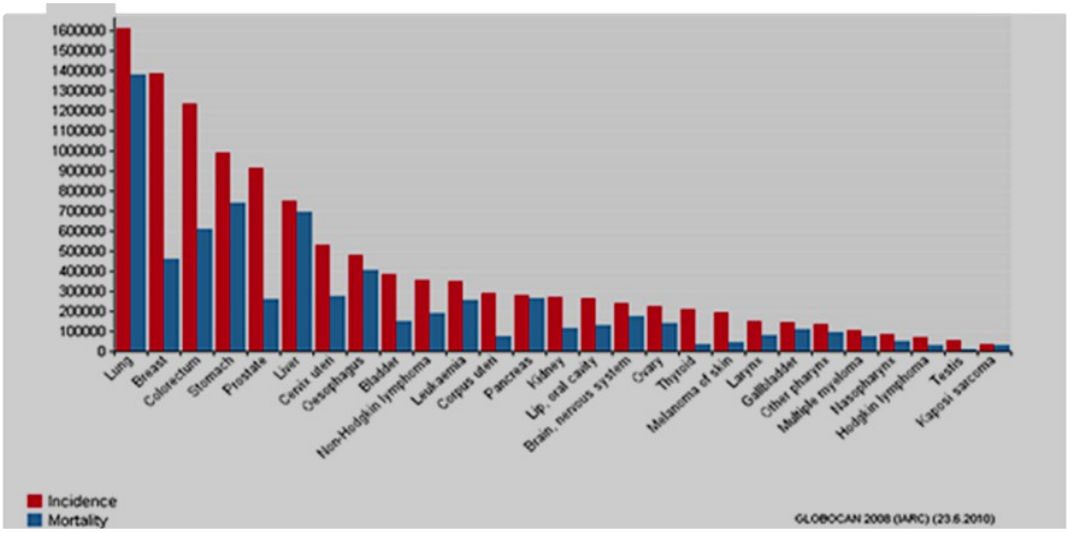


Figure 1 Incidence and mortality of the most common cancers in the world (Data from Globocan 2008)

About the distribution of cancer between the sexes, in the European Region (EURO), as in the rest of the world, the commonest incident cancer for men is lung cancer followed by prostate, colorectal, bladder and stomach cancer. They are also the commonest forms of cancer death in men (**Fig. 2**). Among women breast cancer is the commonest form of cancer and it is also the main cancer cause of death in women, followed by colorectal cancer, lung cancer and stomach cancer (**Fig. 2**).

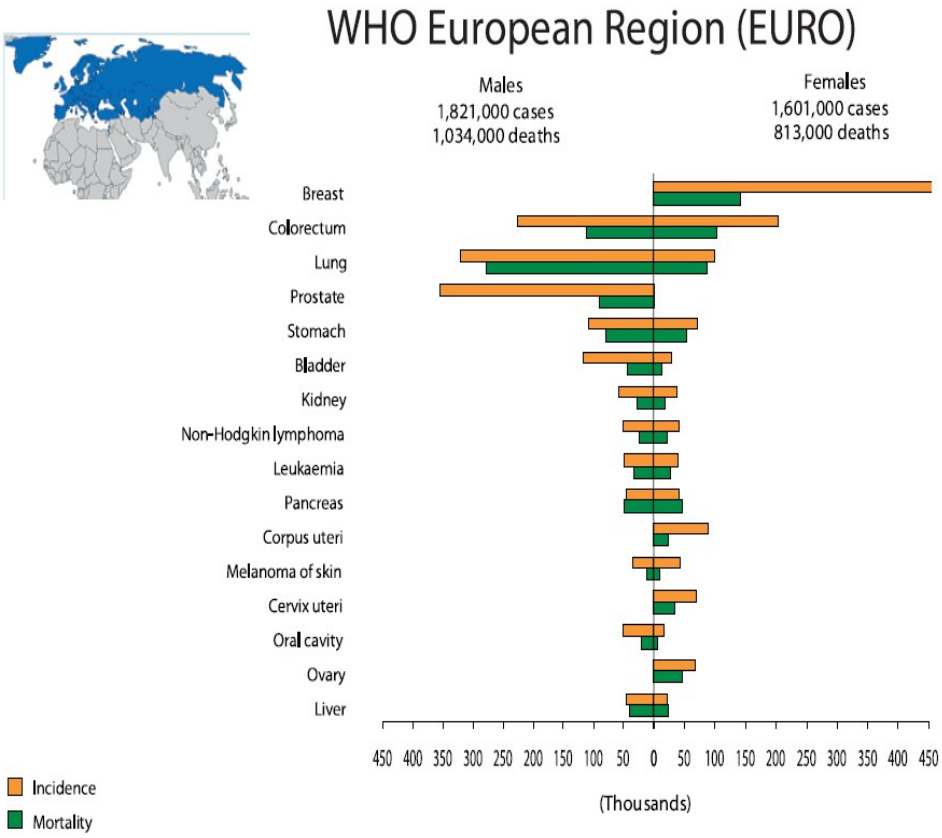


Figure 2 Cancer Incidence and Mortality in World Health Organization European Region (EURO) (Data from World Cancer Report 2008)

2.Breast cancer

2.1 Breast Cancer Current Statistics

Breast cancer is the formation of a malignant tumor that has developed from cells in the breast. A malignant tumor is a group of cancer cells that may grow into (invade) surrounding tissues or spread (metastasize) to distant areas of the body. The disease occurs almost entirely in women, but men can get it, too.

It is the most commonly occurring cancer in women, comprising almost one third of all malignancies in females. It is second only to lung cancer as a cause of cancer mortality, and it is the leading cause of death for American women between the ages of 40 and 55 .

The lifetime risk of a woman developing invasive breast cancer is 12.6 % - one out of 8 females in the United States will develop breast cancer at some point in her life . The American Cancer Society's most recent estimates for breast cancer in the United States are for 2012:

• About 226,870 new cases of invasive breast cancer will be diagnosed in women.

• About 63,300 new cases of carcinoma in situ (CIS) will be diagnosed (CIS is non-invasive and is the earliest form of breast cancer).

• About 39,510 women will die from breast cancer

From the 1940's until recently, the rate of new cases of breast cancer in the United States increased by a little over one percent a year. In the 1980's, the rate of new cases rose markedly (likely due to increased screening) and during the 1990's, the rate of new cases leveled off. Since 2003 there has been a marked decline in the rate of new breast cancer cases . This large decrease was thought to be due to the decline in use of hormone therapy after menopause that occurred after the results of the Women's Health Initiative were published in 2002. This study linked the use of hormone therapy to an increased risk of breast cancer and heart diseases. These decreases are believed to be, also, the result of earlier detection through screening and increased awareness, as well as improved treatment. Incidence rates have been stable since 2004. Rates of breast cancer around the world vary a great deal, with industrialized countries generally having higher rates than non-industrialized countries (**Fig. 3**). And, although all the factors responsible for this variation aren't known, differences between such countries in lifestyle and reproductive factors are thought to play a large role .

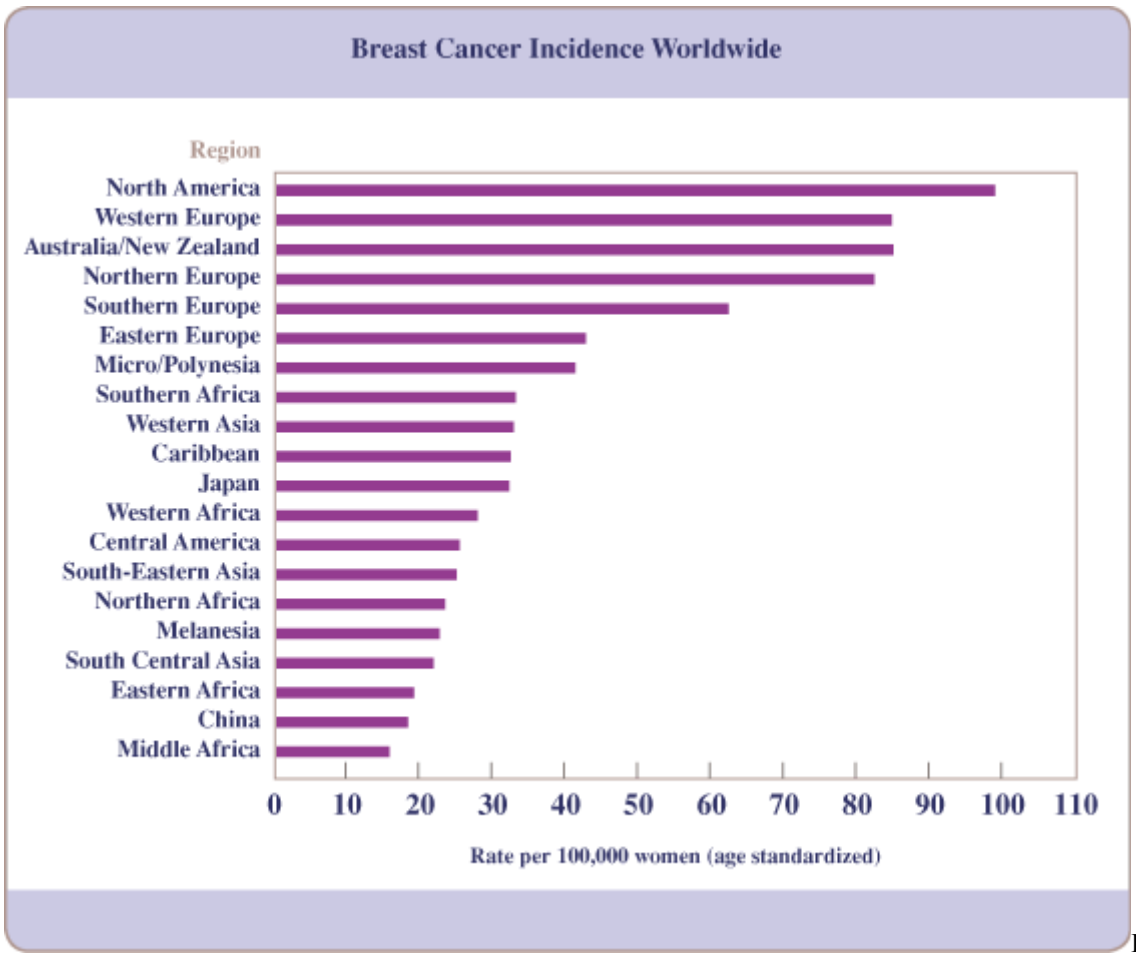


Figure 3 Breast cancer incidence (Data from American Cancer Society 2008).

2.2 The normal breast

In adults, each mammary gland is composed of fifteen to twenty sections called lobes, divided by adipose tissue. Each lobe is subdivided into lobules, which contain the glandular alveoli that secrete the milk into a series of secondary tubules.

These tubules converge to form a series of mammary ducts, which in turn converge to form a lactiferous duct that drains at the tip of the nipple (**Fig. 4**). Fatty tissue and connective tissue surrounding the ducts and lobules, blood vessels, and lymphatic vessels (stroma) .

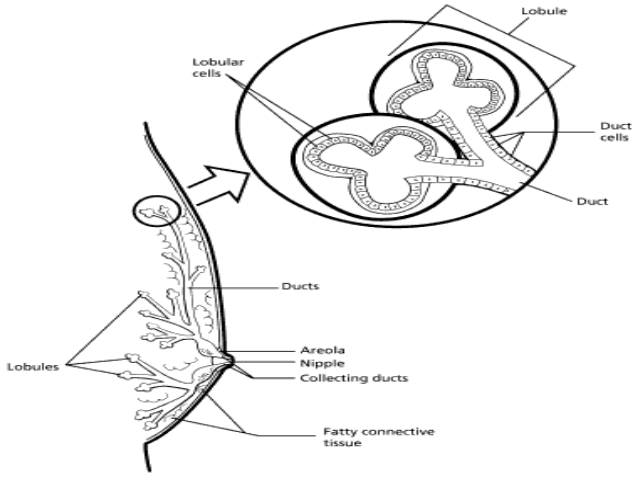


Figure 4 Anatomy of the breast

2.3 Breast Cancer Types

The most common type of breast cancer is ductal cancer. It is found in the cells of the ducts. Cancer that starts in lobes or lobules is called lobular cancer. It is more often found in both breasts than other types of breast cancer. Cancers also are classified as non - invasive (in situ) and invasive (infiltrating). The term *in situ* refers to cancer that has not spread past the area where it initially developed. Invasive breast cancer has a tendency to spread (metastasize) to other tissues of the breast and/or other regions of the body. A less common type of breast cancer is inflammatory breast cancer characterized by general inflammation of the breast.

Other rare types of breast cancer are medullary carcinoma (an invasive breast cancer that forms a distinct boundary between tumor tissue and normal tissue), mucinous carcinoma (formed by the mucus-producing cancer cells), tubular carcinoma, etc .

2.4 Breast Cancer Risk Factors

Every woman is at risk for developing breast cancer. Several relatively strong risk factors for breast cancer that affect large proportions of the general population have been known for some time. However, the vast majority of breast cancer cases occur in women who have no identifiable risk factors other than their gender .

The “established” risk factors for breast cancer are female gender, age, previous breast cancer, benign breast disease, hereditary factors (family history of breast cancer), early age at menarche, late age at menopause, late age at first full-term pregnancy, post

menopausal obesity, low physical activity, race/ethnicity and high-dose exposure to ionizing radiation early in life.

The “speculated” risk factors for breast cancer include never having been pregnant, having only one pregnancy rather than many, not breast feeding after pregnancy, use of postmenopausal estrogen replacement therapy or postmenopausal hormone (estrogen/progestin) replacement therapy, use of oral contraceptives, certain specific dietary practices (high intake of fat and low intakes of fiber, fruits, and vegetables, low intake of phytoestrogens), alcohol consumption, tobacco smoking, and abortion.

Although men can and do develop breast cancer, the disease is 100 times more likely to occur in a woman than in a man . Women are at a higher risk of breast cancer because they have much more breast tissue than men do. Also, estrogen promotes the development of breast cancer. The risk of breast cancer is higher in middle-aged and elderly women than in young women . This risk increases as a woman ages, rising sharply after the age of 40. In 6 the United States, more than three-fourths of all breast cancers occur in women aged 50 or older. A woman who has previously had breast cancer has a three- to four-fold increased risk of developing a new cancer in the other breast. Women who have had benign breast problems are also at increased risk but to a lesser extent .

The risk of breast cancer is higher among women who have a close blood relative (mother, sister, or daughter) who have had the disease. The increase in risk is especially high if the relative developed breast cancer before the age of 50 or in both breasts .

However, most women who get breast cancer (approximately 80 percent) have no such family history of the disease . The effect of family history on breast cancer risk is believed to be due primarily to genetic factors. As much as 5–10 percent of all breast cancer cases are attributable to specific inherited single-gene mutations, and many other cases have some genetic component. The evidence from individual families in which breast cancer occurs very frequently and from large epidemiological studies has shown that some women have a familial predisposition to breast cancer. Epidemiological studies have shown that in women with a family history of breast cancer, the risk of breast cancer is increased two- to threefold. Studies have also shown that there are families in which breast cancer risk is inherited in an autosomal-dominant fashion (‘hereditary breast cancer’). Recently, it has been shown that germline mutations in the BRCA1 and BRCA2 genes account for a large proportion of cases of hereditary breast cancer . Histopathological findings and careful autopsy examinations have played a major role in the recognition of many familial cancer syndromes .

In addition mutations in the BRCA1 and BRCA2 genes, there are as yet unidentified genetic defects that predispose to breast cancer development , and additional studies may help in identifying these genes in the future. Women who reach menarche at a relatively early age (12 or younger) and those who reach menopause at a relatively late age (55 or older) are slightly more likely than other women to develop breast cancer These relationships are believed to be mediated through estrogen production . During the reproductive years, a woman's body produces high levels of estrogen. Women who start to menstruate at an early age and/or reach menopause at a late age are exposed to high levels of estrogen for more years than are women who have a late menarche or early menopause. Age at first pregnancy is another aspect of reproductive history that that is associated with breast cancer risk. Women who have their first full-term pregnancy at a relatively early age have a lower risk of breast cancer than those who never have children or those who have their first child relatively late in life .The biologic basis for this relationship is not entirely clear. Obesity has been consistently associated with an increased risk of breast cancer among postmenopausal women. This relationship may be mediated again by estrogen production. Fat cells produce some estrogen and obese postmenopausal women, therefore, tend to have higher blood estrogen levels than lean women. Studies have consistently shown that the risk of breast cancer is lower among physically active premenopausal women than among sedentary women .

Physical activity during adolescence may be especially protective, and the effect of physical activity may be strongest among women who have at least one full-term pregnancy. Studies of racial/ethnic characteristics of breast cancer reveal that non-Hispanic white, Hawaiian, and black women have the highest levels of breast cancer risk. Other Asian/Pacific Islander groups and Hispanic women have lower levels of risk. Some of the lowest levels of risk occur among Korean and Vietnamese women .

Women who were exposed to high doses of radiation, especially during adolescence, have an increased risk of breast cancer. This association has been observed both among atomic bomb survivors and among women who received high-dose radiation for medical purposes .

Parity (having children) and the age of the woman at the birth of her first offspring are other endogenous hormonal factors that influence breast cancer. Women who have never had children (nulliparous) are at greater risk for the development of breast cancer than women who have had children (parous). There is also consistent evidence that first pregnancy completed before age 30-35 lowers risk of breast cancer, and that first full-term pregnancy after age 30-35 raises risk. More limited evidence suggests that women

who have many pregnancies may be less likely to develop breast cancer than those who have only one pregnancy .Some studies have shown that women who breast-feed their babies may be less likely to develop breast cancer than those who have children but do not breast-feed [22]. Other studies, however, indicate that there may be little or no relationship between breast feeding and breast cancer risk. If breast-feeding does protect against breast cancer, it may do so by delaying the resumption of ovulation (with its accompanying high estrogen levels) after pregnancy. The long-term (more than five years) use of postmenopausal estrogen therapy (ERT) or combined estrogen/progestin hormone replacement therapy (HRT) may be associated with an increase in breast cancer risk . The associations between the use of oral contraceptives and breast cancer have been studied. Many studies attempting to link oral contraceptives with increased breast cancer have been inconclusive .

But these studies have shown that oral contraceptives do not have a large effect on breast cancer risk. Whether they have a small effect on risk is less clear. A possible relationship between breast cancer and diet has been suggested due to the variation of breast cancer in societies with different national diets (the high rates in Western industrialized nations and the low rates in Asia, Latin America, and Africa). A comparison of vegetarian versus meat-eating women produced inconclusive results. No relation between breast cancer risk and total fat, saturated fat, or cholesterol was found. Some of the effects that were once attributed to dietary fat intake were probably due to obesity (which is often linked with high fat intake) rather than to fat intake *per se*. And the effects of fiber, fruits, and vegetables now appear to be small, at best. Diets high in fruits and vegetables and low in fat and calories are healthful for many reasons, and they may indirectly reduce the risk of breast cancer by helping to prevent obesity. Plant substances called isoflavones (sometimes referred to as phytoestrogens) are most commonly found in soy products. It has been speculated that these substances may be 10 protective against breast cancer .

They appear to have effects similar to those of estrogen in some tissues while antagonizing the effects of estrogen in other tissues. A positive, but modest association between alcohol use and breast cancer risk is seen in most studies .

There is also some evidence that cigarette smoking may be associated with a small increase in breast cancer risk. However, epidemiological studies have variably shown positive, inverse, or null associations . Among women who have already been diagnosed with breast cancer, smoking may be associated with an increased risk that the cancer will progress more rapidly. In some studies, premature termination of pregnancy appears to increase breast cancer risk .

In incomplete pregnancy, the breast is exposed only to the high estrogen levels of early pregnancy and thus may be responsible for the increased risk seen in these women. However, some other studies found no association between abortions and increased risk of breast cancer .

2.5 Stages of breast cancer

The staging systems currently in use for breast cancer are based on the clinical size and extent of invasion of the primary tumor (T), the clinical absence or presence of palpable axillary lymph nodes and evidence of their local invasion (N), together with the clinical and imaging evidence of distant metastases (M). This is then translated into the TNM classification which has been subdivided into Stage 0 called carcinoma in situ (lobular carcinoma in situ (LCIS) and ductal carcinoma in situ (DCIS) and four broad categories by the Union Internationale Centre Cancer (UICC), which are the following.

- Stage I – early stage breast cancer where the tumor is less than 2 cm across and hasn't spread beyond the breast .
- Stage II – early stage breast cancer where the tumor is either less than 2 cm across and has spread to the lymph nodes under the arm; or the tumor is between 2 and 5 cm (with or without spread to the lymph nodes under the arm); or the tumor is greater than 5 cm and hasn't spread outside the breast.
- Stage III – locally advanced breast cancer where the tumor is greater than 5 cm across and has spread to the lymph nodes under the arm; or the cancer is extensive in the underarm lymph nodes; or the cancer has spread to lymph nodes near the breastbone or to other tissues near the breast.
- Stage IV – metastatic breast cancer where the cancer has spread outside the breast to other organs in the body (**Figure 5).**

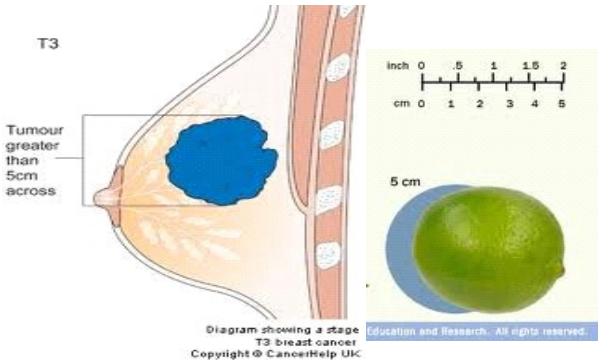


Figure 5 Tumor size stages

2.6 Tumor Markers

A tumor marker is defined as a substance present/overexpressed in or produced by a tumor (tumor-derived), or the host (tumor-associated), that can be used for differentiating neoplastic from normal tissue. Tumor markers are found in cells, tissues, and body fluids such as cerebrospinal fluid, serum, plasma, and milk. Some widely used tumor markers include: AFP, Her2/Neu, beta-HCG, CA 19-9, CA 27.29 (CA 15-3), CA 125, CEA, and PSA. Some tumor markers are associated with many types of cancer; others, with as few as one. Some tumor markers are always elevated in specific cancers; most are less predictable. However, no tumor marker is specific for cancer and most are found in low levels in healthy persons, or can be associated with non-neoplastic diseases as well as cancer. Tumor markers have been categorized as enzymes, isoenzymes, hormones, specific cell membrane proteins, oncofetal and cell-specific antigens, carbohydrate epitopes, oncogene products, genetic changes, etc. There are only a handful of well-established tumor markers that are being used by physicians. Many other potential markers are still being researched. There are many studies now that are trying to find new genes involved in signaling molecules or proteins that “tell” cells to proliferate, invade or metastasize.

HER-2/neu is an oncogene-encoded growth factor receptor (homologue of epidermal growth factor (EGF) receptor), also known as c-erbB-2. It is overexpressed in breast cancers as a result of HER 2 proto-oncogene amplification. It is measured in the tissue from a biopsy either by immunological assays of the protein or PCR. The presence of HER-2/neu is generally associated with a more aggressive growth and poorer prognosis for breast and ovarian cancer .

2.7 The importance of HER2 in breast cancer

The human epidermal growth factor receptors (HER), also known as ERbB receptors, are a family of signal transduction proteins, with partial homology that normally regulate cell growth and survival, as well as adhesion, migration, differentiation, and other cellular responses. There are 4 family members in humans, HER1, HER2, HER3, and HER4 composed of a cysteine-rich extracellular region, a lipophilic transmembrane segment, an intracellular domain with tyrosine kinase activity and a carboxy terminal domain that is autophosphorylated upon receptor activation (**Fig 6**). HER-family receptors become active upon interaction with ligand and subsequent dimerization, followed by activation of downstream signaling proteins by phosphorylation. In humans, the 4 HER proteins interact with a range of ligands, although HER2 has no known activating ligands . For example, HER1 interacts with at least 6 ligands: epidermal growth factor (EGF), transforming growth factor α (TGFα), amphiregulin, heparin-binding EGF-like growth factor, betacellulin, and epiregulin . HER2, also referred to as ERBB2, NEU, or HER2/neu, can act as a dimerization partner with the other HER receptor proteins. As there are no known activating ligands for HER2, its activity is a consequence of its dimerization with other HER-family receptors as well as its homodimerization. Upon activation, the tyrosine kinase domain of each of the HER-family receptors can activate downstream signaling molecules, such as those in the PI3K/Akt and RAS/RAF/MEK/MAPK pathways .

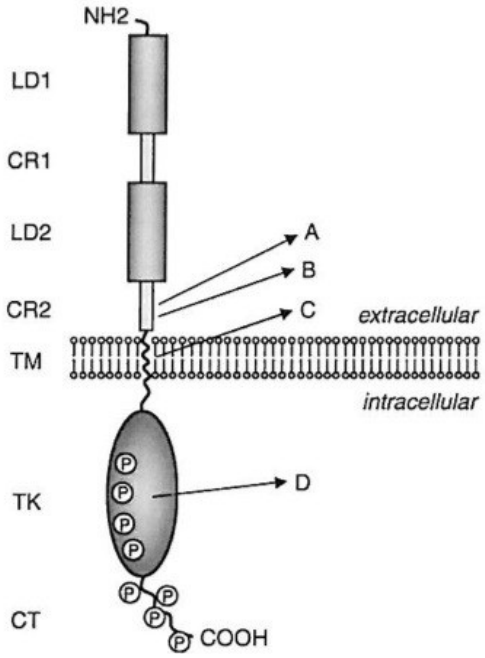


Figure 6 Structure of HER2

The HER2 receptor, a 185 kDa transmembrane glycoprotein, is encoded by the HER2 gene, a protooncogene located on the long arm of chromosome 17q21 . Because of its function as an activator of signaling pathways, HER2 plays a central role in a number of cellular processes, including proliferation, motility, and resistance to apoptosis. As mentioned above, HER2 has no known ligand and can heterodimerize with other HER proteins, thus allowing HER2 to participate in a number of signal transduction pathways in the absence of a specific ligand.

This effect may be enhanced by the overexpression of HER2 in cancer cells, leading to increased cell proliferation and decreased cell death, as well as changes in cell motility. The association of HER2 protein overexpression or HER2 gene amplification with cancer, notably with breast cancer, was reported over 20 years ago.

HER2 is normally expressed at low levels in adult cell types including the breast epithelium, the central nervous system, bone, muscle, skin, heart, lungs and intestinal epithelium. The function of HER2 in adult tissues is not fully understood, however the receptor appears to play a role in the proliferation and differentiation of epithelial cells and in the protection of cardiomyocytes against apoptosis . In fetal tissues, however, HER2 is widely expressed and is critical to normal development. The clinical significance of HER2 in cancer was first discovered in the early 1980s following the identification of the *neu* onocogene, the mutationally active rat homologue of HER2 . The human homologue was soon identified and found to be overexpressed in a mammary carcinoma cell line [37]. On the basis of these findings, Slamon and co-workers examined HER2 expression in a series of primary human breast tumours and reported a significant association between HER2 overexpression, relapse and patient survival . HER2 is now known to be overexpressed in approximately 20-30% of BCs , and overexpression is also common in ovarian, prostate, lung, gastric and oral cancers .

Overexpression of HER2 is a combined result of increased transcription and protein translation. Indeed, breast cancer cells may have as many as 100copies of the gene per cell compared with two copies of the HER2-gene innormal cells [31]. Moreover, there are approximately 20 thousand receptors per cell on normal cells, but breast cancer cells may contain as many as 500 thousand to 2 million HER2 receptors per cell. At this high level of HER2 receptor expression, the kinase activity of HER2 becomes constitutively activated which appears to exert potent mitogenic and transforming effects on cells (**Fig. 7**).

This high density of HER2 promotes the formation of HER2 heterodimers as well as the formation of ligand-independent constitutively active HER2 homodimers . HER2-containing dimers persist longer on the cell surface due to their slower rate of internalization, resulting in overactive cell 18 signaling and leading to dysregulated cell growth, proliferation and malignant transformation [37]. Overexpression of the HER2 receptor is associated with poor prognosis in patients with breast cancer, as well as with aggressive tumor growth and metastases. HER2 positivity has also been associated with tumor grade, 1, 6 positive lymph node metastases at presentation, 1 and mitotic count . HER2+ tumors are less likely to be hormone receptor (estrogen receptor [ER] or

progesterone receptor [PgR]) –positive. HER2 status also correlates with relative response to various agents. HER2 positivity may result in increased resistance to endocrine therapy and with a decreased benefit from non-anthracycline, non-taxane–containing chemotherapy. Conversely, HER2-positive patients may exhibit improved response to anthracycline therapy, as well as to paclitaxel .Data surrounding the prognostic and predictive value of HER2 status are continually evolving. In a recent study, it was shown that higher levels of HER2 gene amplification were associated with worse outcomes in patients treated with doxorubicin-based therapy in the adjuvant setting. The association of HER2 gene amplification or HER2 overexpression with some breast cancers has allowed for the development of agents that specifically target HER2, altering the treatment landscape for these cancers. Trastuzumab, which was approved for the treatment of metastatic breast cancer in 1998, for the adjuvant treatment of lymph node–positive breast cancer in 2006, and for the adjuvant treatment of lymph node–negative breast cancer in 2008, is a umanized monoclonal antibody to the HER2 protein. Lapatinib is a selective inhibitor of the tyrosine kinase activity of HER2 and EGFR. Each of these agents has shown efficacy in patients whose tumors are HER2+ .

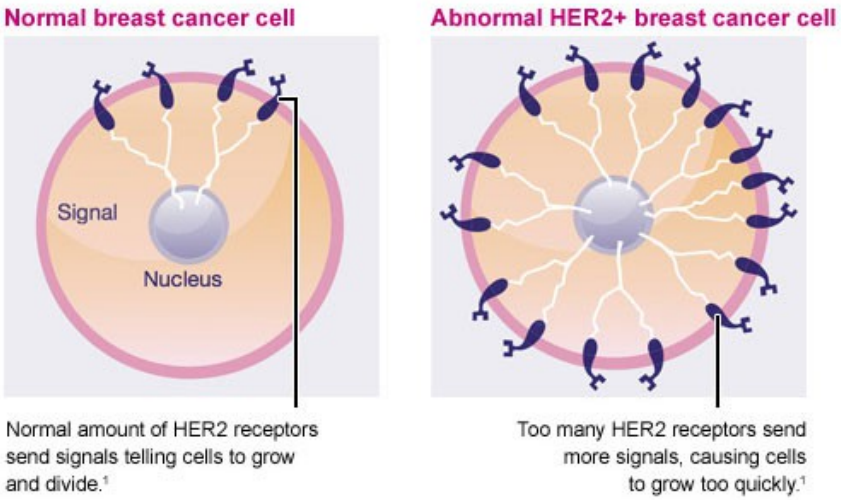


Figure 7 Amount of HER2 in normal and tumoral breast cell

2.8. Trastuzumab Monotherapy

Trastuzumab (Herceptin®, Genentech, Inc., San Francisco, CA), is currently the only HER2-targeted therapeutic agent that has received marketing clearance from the

U.S. Food and Drug Administration (FDA) for use in the treatment of patients with HER2- overexpressing breast cancer .Has been used to treat almost a million women worldwide since 1998. Herceptin given for one year delivers high cure rates in women with HER2-positive early breast cancer. It reduces the risk of cancer coming back by half (50%), as consistently shown by five large studies involving over 15,000 patients and it reduces the risk of dying from breast cancer by a third (34%) Herceptin extends survival across all stages of HER2-positive breast cancer. In advanced (metastatic) HER2-2/4 positive breast cancer, it extends survival by up to four to eight additional months of life on average when used with chemotherapy (Taxol [paclitaxel] and Taxotere® [docetaxel]).Continued treatment with Herceptin prolongs survival without cancer progression in women requiring additional treatment after their cancer had progressed, even though they had already received previous Herceptin therapy. A recent trial has indicated that patients with HER2-positive metastatic breast cancer and central nervous system metastases who receive Herceptin and chemotherapy may experience longer survival than those who do not receive Herceptin therapy. Trastuzumab is a humanized mAb that binds specifically to HER2 on the C-terminal portion of the extracellular domain (ECD) near the juxtamembrane region in domain IV of the receptor. Trastuzumab was constructed by grafting the complementary-determining regions (CDRs) from the murine mAb 4D5 into a human kappa IgG1 to avoid eliciting a human anti-mouseantibody (HAMA) response in patients .

2.8.1 The mechanisms of action

The effectiveness of trastuzumab appears to be correlated with the level of HER2 expression in breast cancer, and with the accessibility of tumors to the drug . However, the mechanism by which trastuzumab induces regression of HER2-overexpressing tumors is not completely understood, but several molecular and cellular effects have been observed in experimental *in vitro* and *in vivo* models. Several of the proposed mechanisms are described below.

2.8.1.1 Immune-mediated response

One of the proposed mechanisms of trastuzumab anti-tumor action is through antibody-dependent cellular cytotoxicity (ADCC) . Specifically, the natural killer (NK) cells are important for the ADCC response to trastuzumab . These cells express the Fcɣ receptor that binds the Fc domain of the IgG1 trastuzumab antibody, and promotes lyses of trastuzumab-bound cancer cells. The importance of this immunological effect was

revealed by Clynes et al. , who achieved a tumor regression rate of 96% in mice bearing HER2-overexpressing BT-474 xenografts treated with trastuzumab, but only a 26% reduction in tumor growth in mice lacking the Fcγ receptor.

2.8.1.2 Inhibition of angiogenesis

Both primary and metastatic breast cancer are dependent on angiogenesis for tumor growth . Trastuzumab can modulate different pro- and antiangiogenic factors to achieve angiogenesis suppression .

2.8.1.3 Inhibition of HER2 extracellular cleavage

The ECD of HER2 can be released by proteolytic cleavage from the fulllength receptor, yielding a 110-kDa fragment that can be detected *in vitro* in cell culture medium, and a 95-kDa amino-terminally truncated membrane associated fragment with increased kinase activity . The HER2-ECD can also be detected *in vivo* in serum, and is currently measured in the clinic withan FDA approved ELISA-based test to follow-up and monitor patients with metastatic breast cancer .Molina et al. demonstrated in HER2-overexpressing SK-BR-3 and BT-474 human breast cancer cells that trastuzumab can block metalloprotease-mediated cleavage of the HER2- ECD. Moreover, several clinical studies have demonstrated that a decline in serum HER2-ECD during trastuzumab treatment correlates with improved tumor responsiveness and progression-free survival , which indirectly supports the hypothesis that trastuzumab may act by inhibiting HER2 cleavage .

2.8.1.4 Inhibition of PI3K pathway

Overexpression of HER2 receptor tyrosine kinases leads to ligandindependent homodimerization and autophosphorylation of tyrosine residues on the cytoplasmic domain of the receptors. Phosphatidylinositol 3' kinase (PI3K) associates with these phosphorylated tyrosine residues and activates downstream effectors, which ultimately leads to enhanced protein synthesis, cell proliferation, survival and motility [64]. Trastuzumab can inhibit the PI3K pathway.

2.8.2. The resistance to trastuzumab

Despite the fact that trastuzumab-based treatment strategy has established a milestone in the therapy of HER-2 positive breast cancer with attractive clinical benefits, either as a single agent or in combination settings, one of the major drawbacks of the

trastuzumab-therappy is trastuzumab resistance, even in highly selected HER-2 overexpressed patients. In fact, only about 30% of HER-2 positive metastatic breast cancer patients respond to trastuzumab and approximately 70% of patients with overexpressed HER-2 receptor may have primary resistance . Additionally, the majority of those patients who achieve initial efficacy tend to develop secondary trastuzumab resistance within one or two years .

Several mechanisms have been postulated in an attempt to explain both intrinsic and acquired resistance to trastuzumab but it is not completely understood.

a) Cleavage of HER-2 extracellular domain to form the truncated HER-2 receptor and the overexpression of membrane associated mucin MUC4 to mask or block the trastuzumab binding site, which can interrupt the interaction between HER-2 receptor and this antibody .

b) Although trastuzumab reduces HER2-mediated signaling, it might not reduce signaling mediated through other HER receptors . For example, heterodimerisation of HER2 with other receptors of the erbB2-family can be induced by ligands of HER1, c) bypass signalling through the non-EGFR family growth factor receptor insulin-like growth factor-1 receptor (IGF-1R), enables activation of the downstream signal cascades without the participation of HER-2. Therefore, the further understanding of trastuzumab actionand resistance mechanisms highlights the need of novel targeted drugs aiming at HER-2 overexpression .

HER3 and HER4, and in the presence of an excess of ligands, the resulting heterodimers may initiate mitogenic signaling even in the presence of trastuzumab .

Indeed, increased levels of the ErbB family ligands EGF and heregulin blocked trastuzumab-mediated growth inhibition of HER2- overexpresssing breast cancer cell lines, and this inhibition was associated with increased signaling from HER2/HER1 (EGFR) and HER2/HER3 complexes .

Valabrega et al. compared tumor tissue from patients with advanced HER2-positive breast cancer before and after trastuzumab treatment, and observed a strong increase in TGFα production upon disease progression, suggesting a possible role of EGFR signaling by TGFα as mediator of acquired resistance to trastuzumab. Indeed, these authors found that trastuzumab was less efficient at inhibiting the growth of HER2-positive SKBR-3 cells engineered to overexpress Tumor Growth Factor alpha (TGFα compared to the parental cells .

3. Mediterranean diet and health status

Cardiovascular diseases and cancer are leading cause of morbidity and mortality in most developed countries. Cancer prevention is therefore an absolute priority for public health, and it should be the obvious choice for financial, social, and above all, human reasons . Cancer and cardiovascular diseases are both known to be fairly refractory to curative interventions. In addition, the occurrence of these diseases exhibits a wide between-country variability and is largely determined by environmental factors and lifestyle habits. Primary prevention through diet and lifestyle is today an established reality to prevent coronary heart disease and stroke, and has achieved an important role in reducing the incidence of, and mortality from, cardiovascular diseases . However, attempts at prevention of cancer have thus far had very modest success . One of the potential answers to overcome this underachievement is related to the growing interest in the Mediterranean dietary pattern as a model for healthy eating and for the primary prevention of cancer. The incidence of certain cancers (e.g. breast and colon cancers) is (or it was) lowest in the Mediterranean area (**Fig. 8**)

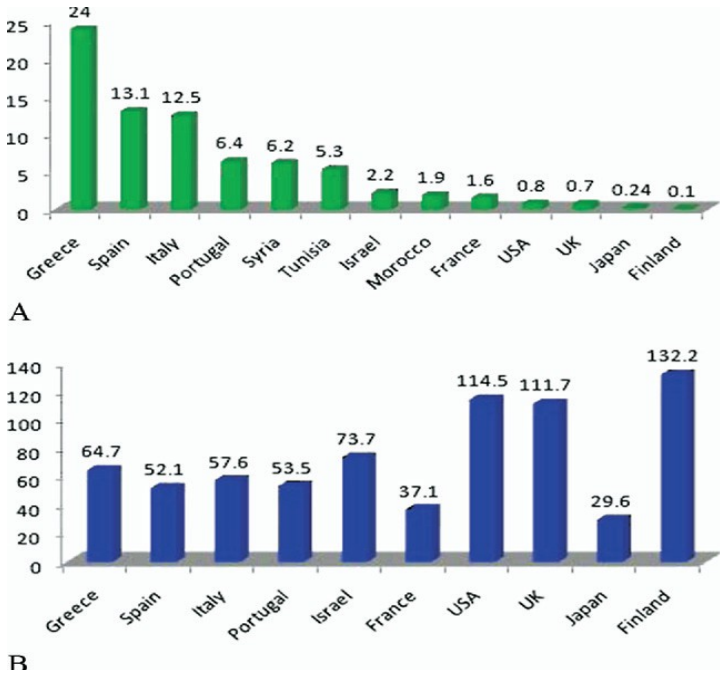


Figure 8 (A) Per capita consumption of olive oil in selected countries (Olive Oil Council Data). (B) Rates of coronary heart disease and cancer mortality according to the World Health Organization standard (Cardiovascular Disease Infobase).

The traditional Mediterranean diet (MD) is the heritage of millennia of exchanges of people, cultures and foods of all countries around the Mediterranean basin. Some 20 countries can be thought of as Mediterranean . Their populations vary in culture, ethnicity, religion, economic development and other factors that can influence dietary intake. Variations on the Mediterranean diet exist in parts of Italy, France, Portugal,

Spain, and elsewhere in the Mediterranean basin, including North Africa and the Middle East . The implication that all Mediterranean people have the same diet is therefore a misnomer, since countries around the Mediterranean basin have different diets, religions and cultures. Their diets vary in the amount of total fat and olive oil consumption, wine intake, type of meat and dairy products, as well as fruit and vegetable. For example, the Italian diet includes a high consumption of pasta, whereas pulses are common in Greece and fish consumption is relatively high in Spain . Moreover, considering the same food item, the nutrient content can vary considerably in different countries. Even in the same country, dietary patterns may be different: for example, dietary patterns are different in northern and southern Italy, the average consumption of cereals, fruit and vegetables being higher in southern Italy, and milk and dairy products in northern Italy . But the dietary patterns that prevail in the Mediterranean region have many common characteristics.

The traditional Mediterranean diet may be thought of as having eight components:

1. High ratio of monounsaturated to saturated dietary lipids (mainly olive oil);
2. Moderate ethanol consumption;
3. High consumption of legumes;
4. High consumption of non-refined cereals, including bread;
5. High consumption of fruits;
6. High consumption of vegetables;
7. Low consumption of meat and meat products;
8. Moderate consumption of milk and dairy products (**Fig 9**).

Figure 9 Mediterranean Diet Foods

All these items are important sources of dietary antioxidants. Apart from the most widely known antioxidants contained in fruits and vegetables, other compounds such as

oleuropein, hydroxytyrosol and other polyphenols present in olive oil, and resveratrol, from red wine, possess a marked antioxidant activity and other advantageous biological properties. The role of free radical production in carcinogenesis together with many epidemiologic studies linking antioxidant intake with a reduced incidence of cancer indicates that dietary antioxidants probably play a protective role. Therefore, the highly palatable traditional Mediterranean diet has many options to be the first choice in the dietary prevention of cancer .

Many epidemiological studies, conducted since the 1980s, investigated the role of the Mediterranean diets on the risk of various chronic diseases. Several aspects of the Mediterranean diet have been related to reduced risks of overall mortality , cardiovascular diseases, as well as several common neoplasms . It has been estimated that up to 25% of colorectal, 15% of breast and 10% of prostate, pancreas and endometrial cancer could be prevented by shifting to a healthy Mediterranean diet .

4. Health benefits of olive oil

Food can be regarded as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutrition, in a way that improves health and well-being or reduces the risk of disease. Extra virgin olive oil (EVOO) could be considered as functional food. Its health properties have been discussed extensively in literature . Different countries and regions in the Mediterranean basin have their own dietary traditions, but in all of them olive oil occupies a central position. The relationship between the intake of olive oil and cancer risk has become a controversial issue that could have very important repercussions in human health as it may have a potential role in lowering the risk of some human neoplasms .

Thus, different studies have shown that the consumption of olive oil have a potential protective effect towards several malignancies, especially breast cancer (stomach, ovary, colon and endometrium cancer too) .

Virgin olive oil (VOO) is a vegetable oil, which can be obtained directly from olive fruit using only mechanical extraction and which can be consumed without further treatments. The chemical composition of olive oil is principally glycerols representing more than 98% of the total weight. Minor components amount to about 2% of the total oil weight and include, among others, aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds, and several antioxidants .

Phenolic compounds represent one of these families of antioxidants which are present in olive oil.

They are of fundamental importance for their nutritional properties, sensory characteristics, and the shelf life of virgin olive oil, since they have high antioxidative properties, can confer a marked bitter taste or a sweet taste typical of some virgin olive oils. They also play an important role in human nutrition as preventative agents against several diseases .

4.1 Phenolic compounds content of olive oils

Among olive oil compounds, the phenolic fraction has received considerable attention in recent years. Evidence from several studies have revealed that the protective effects of EVOO against chronic diseases such as atherosclerosis, cancer, obesity, diabetes, and coronary diseases are related to the phenolic compounds. The pharmaceutical interest in olive oil phenolic compounds due to their bioactivity on different cancer cells is also well known and has stimulated multidisciplinary research on the composition of olive biophenols. The bioactivity of the phenolic compounds in these chronic diseases could be related to different properties such as antioxidant and anti-inflamatory although the molecular mechanism of these compounds in relation to many diseases could have different cellular targets. The most important phenolic compounds that have been identified on EVOO may be divided into different groups such as phenolic acids, phenolic alcohols, secoiridoids, lignans, and flavones (**Fig. 10**).

The main components of the phenolic fraction of EVOO are hydroxytyrosol, tyrosol, and their derivatives linked to the aldehydic and dialdehydic forms of elenolic acid, which are described as secoiridoids (oleuropein, demethyloleuropein, ligstroside). Moreover, significant amounts of lignans, such as pinoresinol and acetoxypinoresinol, are also present, as well as flavonoids (luteolin and apigenin), phenolic acids (such as caffeic, vanillic, syringic, *p*-coumaric, *o*-coumaric, protocatechuic, sinapic and *p*-hydroxybenzoic acid) and hydroxy-isochromans. The exact composition of phenolics compounds in EVOO is related to agronomic and technological aspects .

While the phenolic acids, phenolic alcohols and flavonoids occur in many fruits and vegetables belonging to various botanical families, secoiridoids are present exclusively in plants belonging to the Family of *Olearaceae.* 3,4-Dihydroxyphenyl- ethanol , or hydroxytyrosol, and *p*-hydroxyphenyl-ethanol, or tyrosol are the main phenolic alcohols in VOO (**Fig. 10)**.

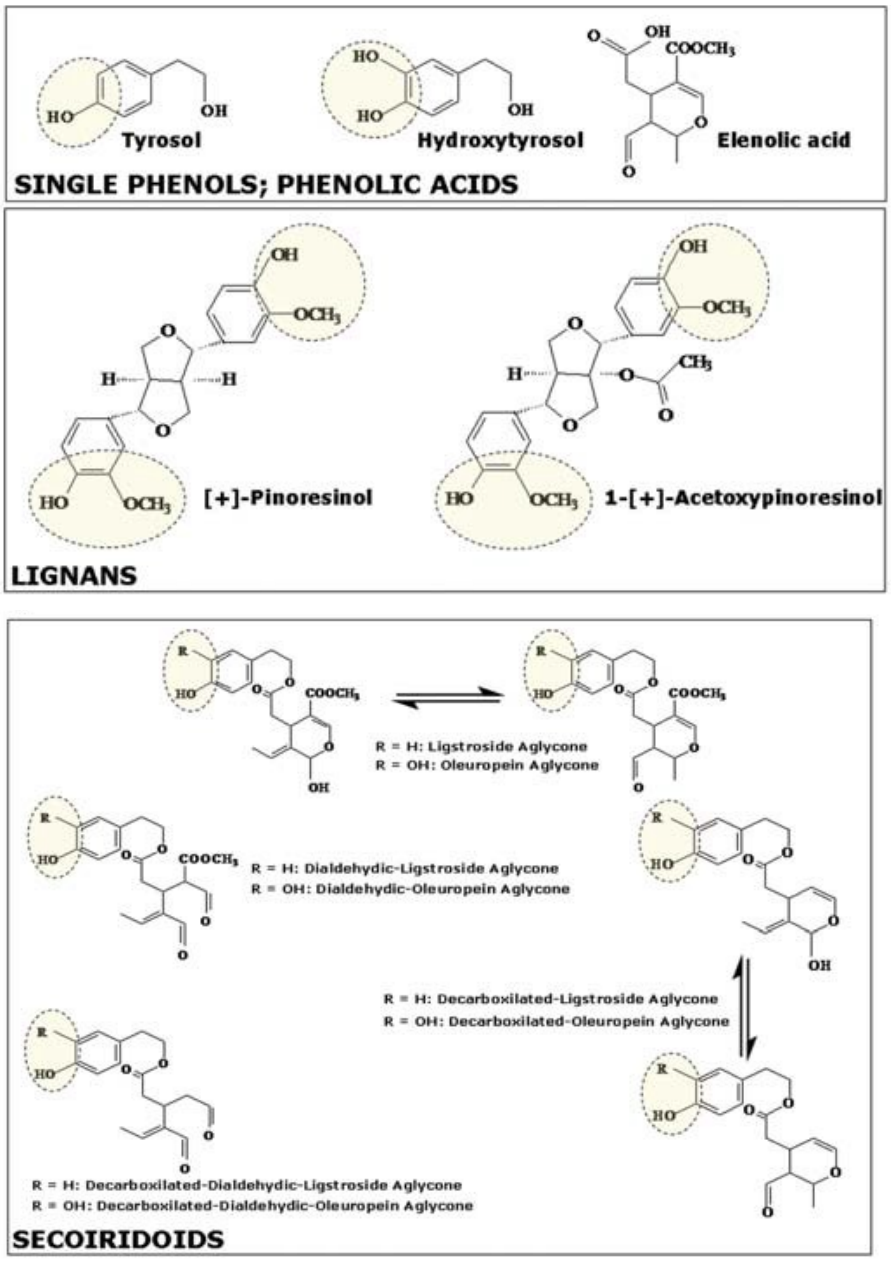


Figure 10

Structures of some EVOO polyphenols

4.2 Health Benefits from phenolic compounds of EVOO

Research has documented a wide variety of anti-inflammatory mechanisms used by olive oil polyphenols to lower our risk of inflammatory problems. These mechanisms include decreased production of messaging molecules that would otherwise increase inflammation (including TNF-alpha, interleukin 1-beta, thromboxane B2, and leukotriene B4); inhibition of pro-inflammatory enzymes like cyclo-oxygenase 1 and cyclo-oxygenase 2; and decreased synthesis of the enzyme inducible nitric oxide synthase . Many different cardiovascular problems - including gradual blocking of the arteries and blood vessels (called atherosclerosis) - have their origin in two unwanted circumstances.

The first of these circumstances is called oxidative stress. Oxidative stress means too much damage (or risk of damage) from the presence of overly reactive oxygen-containing molecules. One of the best ways to help avoid oxidative stress is to consume a diet that is rich in antioxidant nutrients. Many foods contain valuable amounts of antioxidants and anti-inflammatory compounds, but few foods are as rich in these compounds as extra virgin olive oil, and this fact alone accounts for many of the research-based benefits of this culinary oil for health of our cardiovascular system. In terms of antioxidant protection for our blood vessels, olive oil has been shown to lower risk of lipid peroxidation (oxygen damage to fat) in our bloodstream. Many of the fat-containing molecules in our blood - including molecules like LDL - need to be protected from oxygen damage. Oxygen damage to molecules like LDL significantly increases our risk of numerous cardiovascular diseases, including atherosclerosis. Protection of the LDL molecules in our blood from oxygen damage is a major benefit provided by olive oil and its polyphenols .

Several of the polyphenols found in olive oil - including hydroxytyrosol, oleuropein and luteolin - appear to be especially helpful in keeping our blood platelets in check and avoiding problems of too much clumping (called platelet aggregation). There are also two messaging molecules (called plasminogen activator inhibitor-1 and factor VII) that are capable of triggering too much clumping together of the platelets, and the polyphenols in olive oil can help stop overproduction of these molecules .

Benefits of olive oil for the digestive tract were first uncovered in research on diet and cancers of the digestive tract. Numerous studies found lower rates of digestive tract cancers - especially cancers of the upper digestive tract, including the stomach and small intestine - in populations that regularly consumed olive oil. Studies on the Mediterranean Diet were an important part of this initial research on olive oil and the digestive tract. Protection of the lower digestive tract (for example, protection of the colon from colon cancer) is less well-documented in the olive oil research, even though there is some strongly supportive evidence from select laboratory animal studies. Many of these anti-cancer effects in the digestive tract were believed to depend on the polyphenols in olive oil and their antioxidant plus anti-inflammatory properties . One fascinating area of recent research has involved the polyphenols in olive oil and the balance of bacteria in our digestive tract. Numerous polyphenols in olive oil have been shown to slow the growth of unwanted bacteria, including bacteria commonly responsible for digestive tract infections. These polyphenols include oleuropein, hydroxytyrosol, and tyrosol. Some of these same polyphenols - along with other olive oil polyphenols like ligstroside - are

specifically able to inhibit the growth of the *Helicobacter pylori* bacterium. This effect of the olive oil polyphenols may be especially important, since overpopulation of *Helicobacter* bacteria coupled with over-attachment of Helicobacter to the stomach lining can lead to stomach ulcer and other unwanted digestive problems .

The polyphenols found in olive oil are a natural for helping us lower our risk of certain cancer types. Many types of cancer only get initiated when cells are overwhelmed by oxidative stress (damage to cell structure and function by overly reactive oxygen-containing molecules) and by chronic excessive inflammation. Since the polyphenols in olive oil act both as antioxidants and anti-inflammatory molecules, they are perfectly suited for lowering our cells' risk of oxidative stress and chronic unwanted inflammation. Research studies have shown that as little as 1-2 tablespoons of olive oil per day can lower our risk of certain cancer types, including cancers of the breast, respiratory tract, upper digestive tract, and to a lesser extent, lower digestive tract (colorectal cancers) .

While most of the anti-cancer research on olive oil has focused on its polyphenols and their antioxidant and anti-inflammatory properties, several studies have uncovered other fascinating ways in which olive oil provides its anti-cancer benefits. These other ways include the improvement of cell membrane function in a way that lowers risk of cancer development and the altering gene expression in cells in a way that enhances their antioxidant defense system. A final important mechanism linking olive oil intake to decreased cancer risk involves protection of our DNA. The antioxidants in olive oil appear to have a special ability to protect DNA (deoxyribonucleic acids) - the key chemical component of genetic material in our cells - from oxygen damage. DNA protection from unwanted oxidative stress means better cell function in wide variety of ways and provides a cell with decreased risk of cancer development t. There is also encouraging research on the potential for olive oil to help with control of certain cancers once they have already developed. For example, improvement of breast cancer status has been an area of particular interest in olive oil research. Here some of the research has focused on the secoiridoids in olive oil (especially oleocanthal), and its ability to help keep breast cancer cells from reproducing . Another example involves the ability of hydroxytyrosol (HT) in olive oil to trigger programmed cell death (apoptosis) in colon cancer cells . HT may be able to accomplish this anti-cancer effect by helping block the enzymatic activity of fatty acid synthetase (FAS). These cancer-controlling properties of olive oil and olive oil constituents are generally referred to as the "antiproliferative" properties of olive oil.

In numerous laboratory studies, flavonoids have demonstrated the ability to inhibit aromatase activity and thus lower estrogen biosynthesis and circulating estrogen levels, inhibit tumor cell proliferation, and inhibit the formation of reactive oxygen species, all of which are mechanisms thought to influence breast cancer development. Furthermore, dietary intake of certain flavonoids has been reported to potentially protect humans from developing certain types of cancer, including breast cancer .

Several showed that higher dietary intakes of flavonoids and lignans were associated with a reduction in the risk of postmenopausal breast cancers .

Oleuropein has several pharmacological properties, including antioxidant [2], antiinflammatory [36], anti-atherogenic [37], anti-cancer [38], antimicrobial [39], and antiviral [40], and for these reasons, it is commercially available as food supplement in Mediterranean countries. In addition, oleuropein has been shown to be cardioprotective against acute adriamycin cardiotoxicity [41] and has been shown to exhibit anti-ischemic and hypolipidemic activities .

Lignans have anticancer effects in the breast, lung, skin and colon . Antioxidant and antiviral properties of lignans have been reported, and the similarities in structure between some lignans and estradiol or tamoxifen suggest possible activity against breast cancer .

Fini et al. have demonstrated that pinoresinol-rich EVOO have potent chemopreventive properties and specifically is able to decrease the cell viability, to induce apoptosis and modulate cell cycle dynamics in colorectal cancer cell lines.

4.3 Olive oil polyphenols and breast cancer risk

It has been repeatedly suggested that the ability of Mediterranean diet to significantly reduce the risk of several types of human carcinomas including breast cancer , can be largely attributed to the unique healthy characteristics of EVOO, an integral ingredient of the traditional MD. Although these findings might suggest that, in the future, the use of supplements derived from EVOO will be a useful strategy for the prevention and/or treatment of cancer, both the specific components and the specific molecular mechanisms that exert EVOO-related anti-carcinogenic effects have not yet been thoroughly elucidated.

Hydrophilic phenolics represent the most abundant family of bioactive EVOO compounds. As for many plant-derived phenolics, it has been largely assumed that EVOO-derived complex phenols such as secoiridoids (that include aglycone derivatives of oleuropein, dimethyloleuropein and ligstroside, which are also present in olive fruit)

and lignans (such as pinoresinol and acetoxypinoresinol) provide health benefits mainly because of their antioxidant activity . However, the antioxidant capacity of polyphenols does not directly correlate with their efficacy in terms of anticancer activity. Moreover, plasma concentrations of EVOO polyphenols when provided in the diet are often far lower than the levels required for protection against oxidation. It could be argued that metabolites of EVOO polyphenols can reach EVOO-derived compounds, however, tend to have a decreased antioxidant activity compared to parent compound . A large body of evidence indicates that olive oil polyphenols are capable of significantly affecting the overall process of carcinogenesis due to their abilities to inhibit cell cycle, cell proliferation or oxidative stress, improve the efficacy of detoxification enzymes, induce apoptosis, and stimulate the immune system.

Alternatively to general mechanisms largely related to the antioxidant and/or trapping activity of oxygen radicals commonly observed in many plant-derived phenolics, recent studies have demonstrated that complex polyphenols can exert an anti-carcinogenic effect by directly modulating the activities of various types of receptor tyrosine kinases (RTKs) including several members of the HER family . Results studies support the notion that EVOO-derived complex polyphenols may constitute a previously unrecognized family of clinically valuable anti-cancer phytochemicals that significantly affect breast cancer cell proliferation and survival through a molecular mechanism involving the specific suppression of the activity, expression and signal transduction events of the Type I RTK HER2 .

Menendez et al. investigated the anti HER-2 effects of phenolic fractions directly extracted from commercial extra virgin olive oil (EVOO) in cultured human breast cancer cell lines. They tested for the ability to kill both HER-2 positive and negative tumors. The effects of the EVOO fractions on the expression and activation status of HER-2 oncoprotein were evaluated.

They found that all the major EVOO polyphenols (i.e. secoiridoids and lignans) induced strong tumor killing effects by selectively triggering high levels of apoptotic cell death (programmed cell death) in cells over expressing HER-2. The EVOO polyphenols drastically depleted HER-2 protein and reduced autophosphorylation in a dose and time dependent manner. EVOO polyphenols induced HER-2 downregulation regardless of the molecular mechanism contributing to HER-2 overexpression. (i.e.naturally by gene amplification and ectopically driven by a viral promoter).

The researchers concluded that the ability of EVOO derived polyphenols to inhibit HER-2 activity by promoting the degradation of the HER-2 protein itself, together with the fact

that humans have safely been ingesting polyphenols from olive oil for as long as they have been consuming olives and olive oil, support the notion that EVOO is an excellent and safe treatment targeting HER-2.

EVOO-derived phenolics with strong anti-JIMT-1 activity may underscore innovative cancer molecules with novel therapeutic targets because, in order to elicit tumoricidal effects, they should affect the expression and/or activity of genes and transduction cascades closely involved in enhanced cancer cell survival. Importantly, EVOO polyphenols (*i.e.* lignans and secoiridoids) – but not monophenols and phenolic acids – strongly suppressed the growth of breast cancer cells bearing high levels of HER2 (*erb*B2) – one of the most commonly analyzed oncogenes that plays a decisive role in malignant transformation, tumorigenesis andmetastasis in a biologically aggressive subset of human breast carcinomas .

MENENDEZ et al. designed a systematic approach to investigate the tumoricidal and the anti-HER2 effects of EVOO-derived single phenols (i.e. tyrosol and hydroxytyrosol), phenolic acids (i.e. elenolic acid), lignans (pinoresinol and acetoxypinoresinol) and secoiridoids (i.e. deacetoxy oleuropein aglycone -DAOA-, oleuropein aglycone and ligstroside aglycone) directly extracted from EVOO. EVOO-derived lignans and secoiridoids were significantly more effective than EVOO-derived single phenols and phenolic acids to inhibit HER2-associated tyrosine kinase activity strongly suggests that EVOO phenolic compounds with a simple structure (i.e. involving only a single phenol ring) cannot exert inhibitory effects against the tyrosine kinase domain of HER2, and that a more complex structure (i.e. involving two or more phenol rings) is required to efficiently block HER2 tyrosine kinase activity.

Lozano et al. employed crude phenolic extracts (PEs) directly obtained from 14 different monovarieties of EVOOs produced in Spain to preliminary delineate both the biological actions (in terms of cytotoxicity) and the clinical value (in terms of physiologically relevant concentration ranges) of complex multicomponent PEs against HER2 gene-amplified SKBR3 breast cancer cells.

The 14 EVOO varieties had significantly different phenolic compositions, in which secoiridoids were the major phenolic fraction (>90% of total phenolics) in 11 EVOO monovarieties) and lignans were significantly enriched (5-10%of total phenolics) in three EVOO monovarieties. When compared with EVOO PE containing low to undetectable amounts of lignans, their data clearly demonstrated that lignans-enriched EVOO varieties had a relatively weak ability to alter cell viability in the SKBR3 breast cancer model. Thus, the cytotoxic potency of the lignans-negative EVOO-PE 7 (Picual variety from

Cordoba) was found to be 12 times higher than that observed in lignans-enriched EVOO-PE 12 (Arbequina variety from Reus). It should be noted, however, that PE exhibiting small differences in their secoiridoid content notably differed in their abilities to significantly decrease breast cancer cell viability. These findings, altogether, strongly suggest that quality rather than quantity of the entire battery of complex phenols present in individual EVOO-PE ultimately dictate their antibreast cancer cytotoxic effects.

4.4 Limits in the use of bioactive natural compounds

The biological activities of plant polyphenols have been examined by various methods *in vitro* and *in vivo* for prediction of their ability to prevent human diseases. Unfortunately, these valuable natural compounds's uses are substantially limited . It is reported that the polyphenol concentrations needed to obtain in vitro efficiency are generally superior to in vivo moderate levels. According to the route of administration, the efficiency of these compounds depends on their bioavailability and integrity. Indeed, a small proportion of molecules administered orally are absorbed, because of insufficient gastric residence time, low permeability and/or low solubility. Their instability during food processing, distribution or storage, or in the gastrointestinal tract (pH, enzymes, presence of other nutrients), limits the activity and the potential health benefits of polyphenols. The topical use of natural polyphenols is also delicate because of their important sensitivity to environmental factors, including physical, chemical, and biological conditions. Unfortunately, they oxidize very quickly, leading to the progressive appearance of a brown color and/or unwanted odors with a considerable loss in activity. Other problems related to polyphenol use in human health have to be solved. A large number of polyphenolic compounds from natural sources are interesting for their properties, however in their free form, they can show limited water solubility. Furthermore, many polyphenols have an unpleasant taste which must be masked before their incorporation in foodstuffs or oral medicines. Therefore, the administration of phenolic compounds requires the formulation of a finished protecting product able to maintain the structural integrity of the polyphenol until the consumption or the administration, mask its taste, increase its water solubility and bioavailability, and convey it precisely towards a physiological target. Among the existing stabilization methods, encapsulation is an interesting means. The use of encapsulated polyphenols instead of free compounds is the source of numerous works.

5. Drug Delivery System

Drug delivery systems (DDSs) can improve the pharmacological properties of traditional drugs by altering drug pharmacokinetics and biodistribution .

DDS can include liposomes, nanospheres, micelles, dendrimers, as well as various polymeric-based systems . Among the many DDS available, liposomes gained popularity in recent years thanks to their clinical success.

Due to their small size (about 100 nm or less), they readily extravasate from circulation through vascular gaps or defects attributed to ongoing angiogenesis that is typical of tumour sites , which have been reported to be about 200 nm or greater. DDS retention within these sites is generally high due to the poor lymphatic drainage observed within tumors . Furthermore, their lower size limit of diameter ensures that these vehicles do not randomly penetrate normal vessel walls. In cancers, an imbalance in factors that regulate angiogenesis, such as overexpression of, results in both increased vascular permeability and chaotic tumour-vessel architecture. In combination, these effects cause enhanced permeation and retention (EPR), resulting in high local drug concentrations.

5.1 Liposomes

Liposomes are spherical, self-closed structures formed by one or more concentric lipid bilayers containing an aqueous phase inside and between the bilayers (**Fig. 11 A**).

The lipids used in the formation of liposomes are usually comprised of a hydrophilic headgroup and two hydrophobic fatty acyl chains. These amphiphilic molecules spontaneously assemble into aggregates in an aqueous environment. Phosphatidylcholine (PC), also called lecithin, is a biocompatible phospholipid that exists in plants and animals and used frequently in liposomal preparation. Moreover, there are other molecules widely used in combination with phospholipids, such as cholesterol.

The exact location of a drug in liposomes will depend upon its physicochemical characteristics and the composition of the lipids.

However, as a general rule, the hydrophilic drug molecules can be encapsulated in the aqueous space whereas the hydrophobic and amphiphilic molecules can be incorporated into the lipid bilayer (**Fig. 11 B**).

A **B**

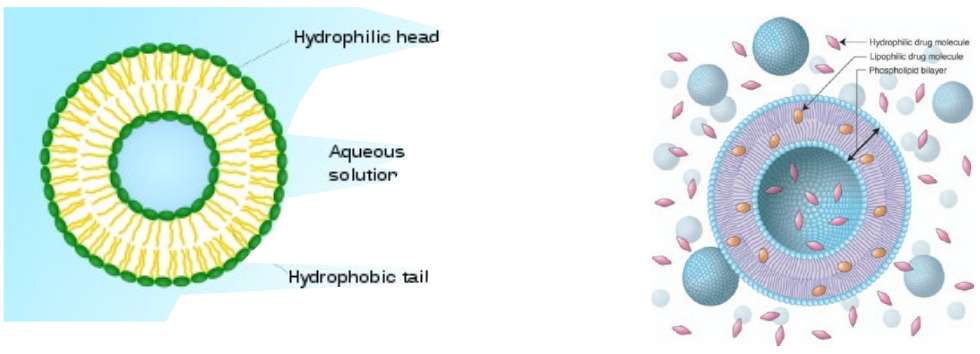


Figure 11 A Schematic illustration of a liposome. **B** Types of drugs and site of their incorporation into liposoma

Numerous evidences have demonstrated the ability of liposomes to enhance the efficiency of drug delivery via several routes of administration . One of the major effects of liposomes as drug carriers is altering the pharmacokinetics of drug. It is known that pharmacological response is dependent upon the concentration of the drug in 24 the target cell. The drug concentration in the target site is governed by absorption, distribution and elimination. These processes may influence the pharmacokinetics of the drug and lead to inefficient utilization of the therapeutic agent. Thus, higher doses need to be administrated. Furthermore, higher drug doses often lead to resistance and undesirable immunological and toxicological effects .

Liposomes are thought to shield all or most of the drug molecules resulting in decreasing the direct contact of drug with biological environment, thus the pharmacokinetic profile of the drug will be determined by the physiochemical properties of liposomes, rather than the drug itself . Incorporating the drug into a vehicle capable of delivering it intact would overcome many of the disadvantages of the free drug administration.

Improving the pharmacokinetics of the drug by this method could lead to beneficial effects such as reduced dosages, increased cellular permeability and delayed drug elimination .

5. 1. 1 Classification of Liposomes

Liposomes are usually classified according to their lamellarity and size. The following categories show the major types of liposomes:

Multilamellar vesicles (MLV): This population has a broad range of size distribution that occurs in a range of 100-1000 nm. The lipid composition may influence the lamellarity of these MLVs. However, the lamellarity typically varies between 5 and 25 concentric lamellae.

Large unilamellar vesicles (LUV): The size of these vesicles is normally up to 1000 nm and the structure consists of a single lamellae.

Small unilamellar vesicles (SUV): The structure normally consists of single lamellae and the diameter of this population is below 100 nm.

The choice of preparation method can influence the size and lamellarity of liposomes, resulting in different characteristics and fate of entrapped drug in vivo (**Fig 12**).

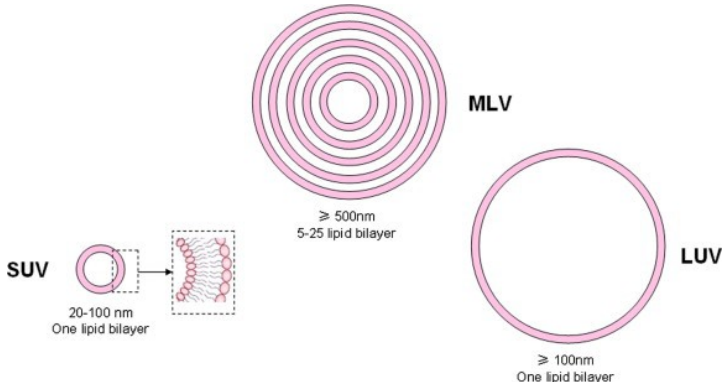


Figure 12 Main classification of liposomed base in the structure

Liposomes were suggested as drug carriers in cancer chemotherapy by Gregoriadis et al. in 1974. Since then, the interest in liposomes has increased and liposome systems are now being extensively studied as drug carriers. Three basic requirements need to be fulfilled if liposomes are to be successful in delivering drugs specifically to cancerous tissue: (i) prolonged blood circulation, (ii) sufficient tumor accumulation, (iii) controlled drug release and uptake by tumor cells with a release profile matching the pharmacodynamics of the drug.

The pharmacokinetics of liposomes depend on their physiochemical characteristics such as size, surface charge, membrane lipid packing, steric stabilization, dose and route of administration . In general, larger liposomes are eliminated from the blood circulation more rapidly than smaller ones . Binding of opsonins to liposomes depends on liposome size; consequently, the reticuloendothelial (RES) uptake of liposomes by the liver is size-dependent . The action of the reticuloendothelial system results in rapid removal from the blood and accumulation in tissues such as liver and spleen. In general, for a given liposome composition, the larger the liposome, the faster the clearance by the RES .

Optimal liposome size depends on the tumor target. In tumor tissue, the vasculature is discontinuous, and pore sizes vary from 100 to 780 nm. By comparison, normal vascular endothelium is < 2 nm in most tissues, 6 nm in post capillary venules, 40–60 nm for the kidney glomerulus, and up to 150 nm for sinusoidal epithelium of the liver and spleen. Most liposomes are 100 nm .

Negatively charged liposomes were believed to be more rapidly removed from circulation than neutral or positively charged liposomes; later studies have indicated that the type of negatively charged lipid affects the rate of liposome uptake by the RES. For example, liposomes containing negatively charged lipids that are not sterically shielded (phosphatidylserine, phosphatidic acid, and phosphatidylglycerol) are cleared more

rapidly than neutral liposomes of similar composition. However, liposomes containing sterically shielded lipids (for example ganglioside-GM1 and phosphatidylinositol) are cleared even more slowly than neutral liposomes .

Thus, one way to reduce liposomal uptake by the RES is by creating sterically stabilized liposomes. “Steric stabilization” refers to the colloidal stability resulting from attachment of hydrophilic polymers or glycolipids on the liposomes. The best-studied stabilizers are polyethylene glycol and ganglioside GM1. Sterically stabilized liposomes (Stealth) showed prolonged lifetimes in the circulation as compared with nonstabilized Liposomes . Sterically stabilized liposomes are also less reactive toward serum proteins and less susceptible to RES uptake than nonstabilized liposomes . The mechanism by which sterically stabilized liposomes are thought to decrease RES-mediated uptake is that the stabilizer occupies the space immediately adjacent to the liposomal surface, excluding other macromolecules from this space. Consequently, access to and binding of blood plasma opsonins to the liposome surface are hindered, preventing interactions with RES macrophages. However, although sterically stabilized liposomes prolong circulation time and decrease liposomal uptake by the RES, they do not actively target the liposome to the tumor.

Polyethylene glycol (PEG) is the most widely used polymeric steric stabilizer.

PEG is a linear polyether diol with many useful properties. It is highly soluble in aqueous and organic media and possesses very low immunogenicity and antigenicity and is non-toxic. It can be attached to the liposome surface in various ways, but the most widely used method is to anchor the polymer in the liposome membrane via a cross-linked lipid (for example PEG-DSPE). It was shown that steric stabilisation of liposomes with PEG increases their longevity in the circulation . A supplementary method to ensure long circulation times involves adding cholesterol (Chol) to the lipid bilayer.

Cholesterol acts as a spacer between phospholipids of the liposome membrane because of its inflexible structure, preventing demixing of the lipids and reduces PEG chain-chain interactions. This results in improved steric stabilization of the liposomes.

5.2 Immunoliposomes for the target delivery of antitumor drugs

A large variety of therapeutically active molecules (e.g. antitumor drugs, oligonucleotides, DNA, enzymes, peptides and hormones) have been successfully incorporated in liposomes. Especially in the field of cancer chemotherapy, much effort has been invested to successfully achieve site specific drug delivery with liposomal systems. Active targeting of liposomes to tumor cells is generally attempted by

conjugating ligands to the liposomal surface which allow a specific interaction with the tumor cell. A large variety of therapeutically active molecules (e.g. antitumor drugs, oligonucleotides, DNA, enzymes, peptides and hormones) have been successfully incorporated in liposomes. Especially in the field of cancer chemotherapy, much effort has been invested to realize site-specific drug delivery with liposomal systems. Active targeting of liposomes to tumor cells is generally attempted by conjugating ligands to the liposomal surface which allow a specific interaction with the tumor cells . Several types of ligands have been used for this purpose, including antibodies or antibody fragments ,vitamins , glycoproteins and, peptides (RGD-sequences) , and oligonucleotide aptamers .

The first report on antibody-targeted liposomes came from Torchilin et al. exactly 2 decades ago . These antibody-targeted liposomes (further referred to as immunoliposomes) were shown to be able to specifically bind to the antigen that is expressed on the target cells. Since then, several coupling techniques have been described for conjugating antibodies or their fragments to liposomes, each with their own advantages and drawbacks . Many in vitro experiments have demonstrated highly specific binding ofimmunoliposomes to target cells. However, despite the excellent targeting properties in vitro, successful results on targeting of immunoliposomes in tumor models are, up to now, scarce.

5.2.1 Clearance of immunoliposomes from the circulation

Perhaps the most important barrier limiting the usefulness of immunoliposomes for targeted drug delivery has been the rapid recognition and removal from the blood by cells of the mononuclear phagocyte system (MPS), particularly the macrophages in liver and spleen . In addition, the presence of whole antibodies conjugated to the liposomal surface makes immunoliposomes highly susceptible to Fc-receptor-mediated phagocytosis and, as a result, even more prone to rapid clearance . The Fc-receptor family, which is expressed by different cells of the MPS, binds the constant region (Fc) of antibodies resulting in internalization of antibody–opsonized complexes (e.g. bacteria) .

Similarly, Immunoliposomes bearing whole antibodies are cleared rapidly due to exposed Fc parts .

The advent of so-called long-circulating liposomes produced by coating the liposome surface with the polymer poly(ethyleneglycol) (PEG), has revived interest in targeted drug delivery . The PEG-coating has a dual effect. It sterically stabilizes the liposomal membrane against interactions with destabilizing and opsonic factors in vivo. As a

consequence, PEG-coated liposomes show longer circulation times and reduced uptake by the MPS, relative to conventional liposomes. Although the exact mechanism behind the MPS avoidance phenomenon is still under discussion, it is thought that stabilization occurs by the formation of a highly hydrated shield of polymer molecules around the liposome which sterically inhibits both electrostatic and hydrophobic interactions of serum components with the liposomal bilayer . Therefore, PEG–liposomes are often referred to as sterically stabilized liposomes.

There are different methods available for coupling antibodies to PEG–liposomes .

For attaching antibodies to the surface of PEG–liposomes, two main strategies have been followed: those in which the ligand is coupled directly to the liposome bilayer **(Fig. 13 A)** and those in which the ligand is attached to the terminal end of PEG **(Fig. 13 B–D)** . The latter strategy yields protein present at the surface of the PEG coating. Indeed, it has been shown that the clearance rate of PEG–immunoliposomes is dependent on the antibody density at the liposome surface . At low antibody density (<≈50 μg mAb/μmol PL), the PEG–immunoliposomes are cleared at rates only slightly more rapidly than those seen for antibody-free PEG–liposomes. At higher antibody density (>100 μg mAb/μmol PL), the clearance becomes very rapid with half-lives of only a few minutes . Likely, clearance is mediated by the exposed Fc-region of the whole antibodies conjugated to the PEG-terminal ends.

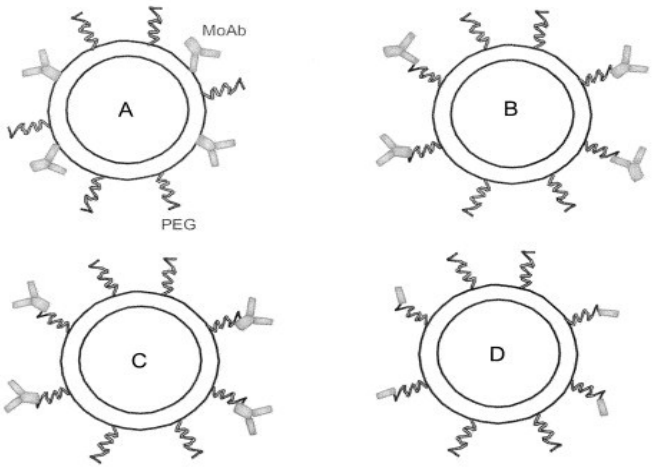


Figure 13 Overview of the currently used types of PEG – immunoliposomes**.**

came from Maruyama and colleagues who investigated whether the use of Fab′ fragments instead of whole antibodies makes a difference in terms of pharmacokinetics and biodistribution of PEG–immunoliposomes .

PEG–immunoliposomes exposing per particle approximately 51 whole antibody molecules conjugated at the distal ends of the PEG-chains were found to be rapidly removed from the circulation (60% after 1 h), whereas the same type of PEG–immunoliposomes, bearing per particle an average of 517 Fab′ fragments derived from the same monoclonal antibody, showed a 6-fold longer circulation time . Moreover, accumulation of Fab′-bearing PEG–liposomes directed against the human carcinoembryonic antigen (CEA) in human gastric cancer tumors in nude mice was 2-fold higher compared to the accumulation of PEG–liposomes bearing whole IgG (approximately 20 and 10% of injected dose/g tissue, respectively) and was comparable with the accumulation of non-targeted PEG–liposomes. These results suggest that either the chemical modification of whole antibody molecules needed for coupling to the liposomes or the Fc-portion of antibody molecules mediate an enhanced clearance rate of antibody-targeted PEG–liposomes. Therefore, the use of Fab′ fragments rather than whole antibodies coupled to the distal ends of PEG-chains seems preferable for directing sterically stabilized liposomes to solid tumors. Nevertheless, many investigators still tend to use whole antibodies as targeting devices for PEG–liposomes.

6. Cellular membrane

Cells interact with their environment in a number of ways. Each cell needs to obtain oxygen and other nutrients (carbohydrates, amino acids, lipid molecules, minerals, etc.) from the environment, maintain water balance with it surroundings, and remove waste materials from the cell. The boundary between any cell and its environment (through which substances must pass) is the membrane, lipids and proteins are the staple ingredients of membranes, although carbohydrates are also important. Scientists began building molecular models of the membrane decades before membranes were first seen with the electron microscope (in the 1950s). In 1915, membranes isolated from red blood cells were chemically analyzed and found to be composed of lipids and proteins. Ten years later, two Dutch scientists reasoned that cell membranes must be phospholipid bilayers. Such a double layer of molecules could exist as a stable boundary between two aqueous compartments because the molecular arrangement shelters the hydrophobic tails of the phospholipids from water while exposing the hydrophilic heads to water. Although the headsof phospholipids are hydrophilic, the surface of a pure phospholipid bilayer adheres less strongly to water than does the surface of a biological membrane. Given this difference, Hugh Davson and James Danielli suggested in 1935 that the membrane might be coated on both sides with hydrophilic proteins. They proposed a sandwich model: a

phospholipid bilayer between two layers of proteins. When researchers first used electron microscopes to study cells in the 1950s, the pictures seemed to support the Davson-Danielli model. By the late 1960s, however, many cell biologists recognized two problems with the model. First, inspection of a variety of membranes revealed that membranes with different functions differ in structure and chemical composition. A second, more serious problem became apparent once membrane proteins were better characterized. Unlike proteins dissolved in the cytosol, membrane proteins are not very soluble in water because they are amphipathic. If such proteins were layered on the surface of the membrane, their hydrophobic parts would be in aqueous surroundings. Taking these observations into account, S. J. Singer and G. Nicolson proposed in 1972 that membrane proteins reside in the phospholipid bilayer with their hydrophilic regions protruding. This molecular arrangement would maximize contact of hydrophilic regions of proteins and phospholipids with water in the cytosol and extracellular fluid, while providing their hydrophobic parts with a nonaqueous environment. In this fluid mosaic model, the membrane is a mosaic of protein molecules bobbing in a fluid bilayer of phospholipids. A method of preparing cells for electron microscopy called freeze-fracture has demonstrated visually that proteins are indeedembedded in the phospholipid bilayer of the membrane (**Fig .14**)

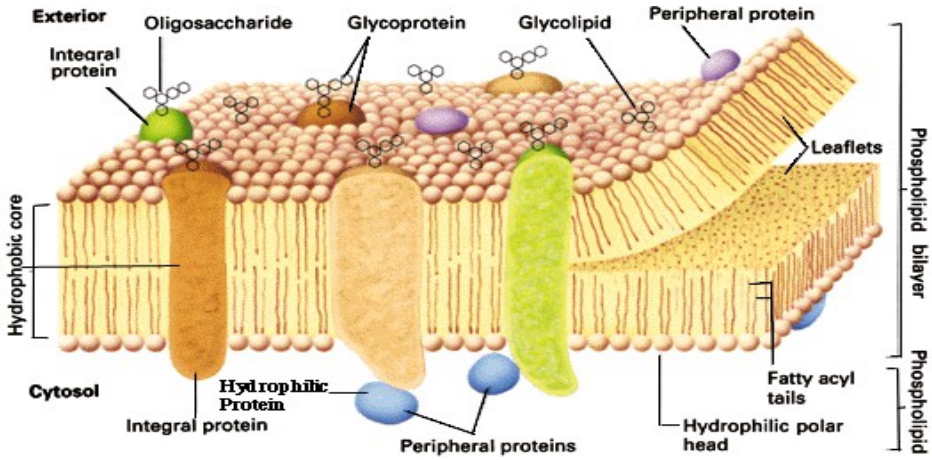


Figure 14 The Singer–Nicholson 'fluid mosaic model'

6.1 Lipid composition of membrane

Biological membranes display a very complex composition in terms of lipids and proteins. Lipid bilayers are non-covalent structures spontaneously formed by lipid molecules due to their amphiphilic nature, that is, they contain a polar region (hydrophilic headgroup), which prefer high dielectric constant (polar) solvents such as water, and hydrocarbon regions (hydrophobic tails), which prefer non-polar solvents such as oil. The

hydrophobic tail is generally composed of one or two fatty acid chains, whereas the head group varies depending on the type of lipid.

Membrane lipids are classified into three main groups, namely phospholipids, glycolipids and sterols (**Fig. 15**)

The main phospholipids found in biological membranes are glycerophospholipids (40-60 mol % of the total lipid fraction).

These compounds are composed of a glycerol backbone on which two fatty acid chains are esterified in position *sn*-1 and *sn*-2, respectively. The third carbon atom of the glycerol backbone (position *sn*-3) supports the phospholipid polar head group, which is composed of an alcohol molecule (choline, ethanolamine, serine, glycerol or inositol) linked to a negatively charged phosphate group. The phospholipid polar head group can be zwitterionic or negatively charged. The fatty acid chain in position *sn*-1 is generally saturated and composed of 16 or 18 carbon atoms while the fatty acid chain in position *sn*-2 is longer and usually unsaturated (one or several double bonds in *cis* configuration) .

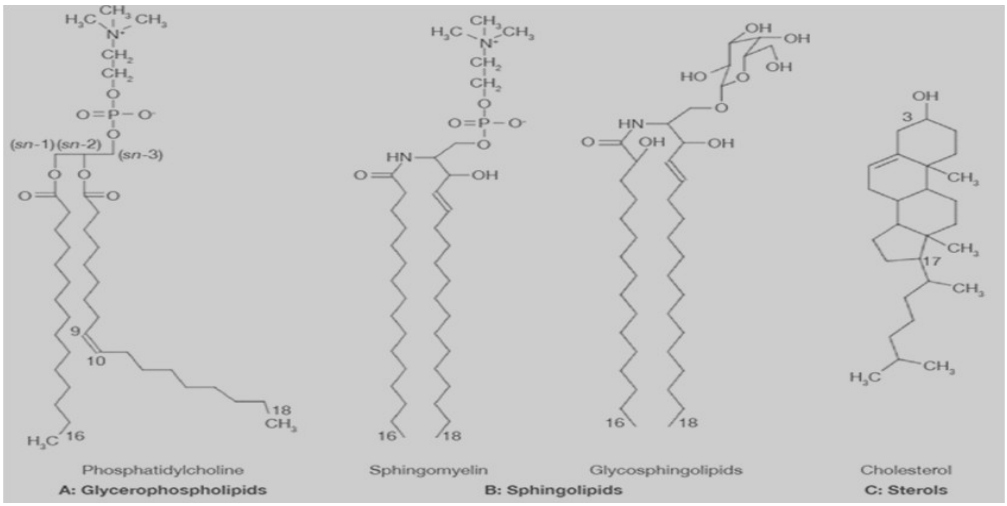


Figure 15. Principales membrane lipids

Phospholipids represent greater than 50 weight % of the total lipid mass in most case, although their relative compositions vary depending on the type of cell (**Table 1)**. The content of charged lipids is roughly 10 mole % in all membranes and is highest in the plasma membranes (11–13%). The plasma membranes of mammalian cells have a high content of cholesterol which amounts roughly to 20 weight % of the total lipid .

	Composition (mol %)			
Source/Location	PC	PE + PS	SM	Cholesterol
Plasma membrane (human erythrocytes)	21	29	21	26
Myelin membrane (human neurons)	16	37	13	34
Plasma membrane (*E. coli*)	0	85	0	0
Endoplasmic reticulum membrane (rat)	54	26	5	7
Golgi membrane (rat)	45	20	13	13
Inner mitochondrial membrane (rat)	45	45	2	7
Outer mitochondrial membrane (rat)	34	46	2	11

Table 1 Major lipid components of selected biomembranes

In human erythrocytes the PLPC molecule is one of the most abundant phosphatidylcholines (PCs) and can be considered as biologically representative. Dimyristoylphosphatidylcholine(DMPC,14:0/14:0)anddipalmitoylphosphatidylcholine(DPPC, 16:0/16:0) are common lipid species in the sense that they have served as a constituent of model membranes in numerous experimental and computational studies aiming at methodological advances and an understanding of membrane structure. These kinds of saturated lipids also occur in biological membranes, albeit as minor species.

In addition to glycerophospholipids, the main components in eucariotic cell membranes are sphingolipids and cholesterol. The most significant sphingolipid in this context is sphingomyelin (SM), a phosphate-containing sphingolipid that together with glycerophospholipids forms the group of phospholipids (FIG 15). Sphingolipids are believed to be involved in the formation of lateral microdomains in biological membranes. These lipids are composed of a sphingosine (or phytosphingosine) base on which is linked a relatively long (up to 24 carbon atoms) saturated fatty acid chain. Acylated sphingosines are referred to as ceramides. Sphingomyelin and glycosphingolipids result from the attachment of a choline molecule and an oligosaccharide to the hydroxyl group of ceramides, respectively. Variations in the cell membrane lipid compositions of many species appear to be evolutionary adaptations that maintain the appropriate membrane fluidity under specific environmental conditions.

Sterols are a particular class of membrane lipids. While the hydrophobic moiety of most of membrane lipids is constituted of relatively long aliphatic chains, the one of sterols is composed of polycyclic structures. The most abundant sterol in mammal is cholesterol (**Fig. 15**). This compound is very abundant in erythrocyte membranes, other plasma membranes and various sub-cellular compartments in eukaryotes (30-50 mol % of the total lipid fraction). It comprises four fused cycles in *trans* configuration, a hydroxyl group in position 3, a double bond between the carbon 5 and 6, as well as an iso-octyl lateral chain in position 17. The hydroxyl group is responsible for the amphiphilic nature of cholesterol and consequently for its orientation in biological membranes. Ergosterol and lanosterol are two other representatives of the sterol class. These compounds exhibit a similar structure to the one of cholesterol. Ergosterol is found in the membranes of fungi, yeasts and protozoans , while lanosterol is the sterol of prokaryotes and the chemical precursor of both cholesterol and ergosterol. Cholesterol is a steroid that is known to maintain membrane structure and regulate membrane fluidity . Membranes with higher cholesterol content have lower plasma membrane fluidity .Cholesterol's chemical structure dictates its orientation within phospholipids; its polar hydroxyl group interacts with phospholipid head groups, while a hydrophobic steroid ring is oriented parallel to the hydrocarbon chains of the phospholipid bilayer . Cholesterol molecules immobilize the first few hydrocarbon groups of the phospholipids making bilayer less deformable. In addition, cholesterol prevents crystallization of hydrocarbons in fatty acid chains because part of its steroid ring is closely attracted to the part of the fatty acid chain on the closest 21phocpholipid. The above structure leads to slight immobilization of the outer surface of the membrane, which makes it less soluble to the small water-soluble molecules, and prevents membrane from being overly fluid. The shape of a membrane lipid depends on the effective area of its polar head group compared to the dimension of its hydrophobic moiety . Membrane lipids display a cylindrical shape (*e.g.* phosphatidylcholine, phosphatidylserine), a conical shape (*e.g.* phosphatidylethanolamine) or an inverted conical shape (*e.g.* lysophosphatidylcholine). Such a polymorphism influences the localization of lipid molecules within the biological membranes. The lipid composition of biological membranes is qualified as asymmetric , *i.e.* the lipid composition is different within the two leaflets of the same membrane. Phosphatidylethanolamines and phosphatidylserines are mainly found in the inner leaflet of the plasma membrane, while phosphatidylcholines and sphingomyelins are essentially located in the outer leaflet. Due to its ability to undergo a fast flip-flop between the outer and inner leaflets of the lipid bilayers ,

cholesterol is assumed to be equally distributed on the two leaflets of biological membranes.

6.2 Membrane alterations and cancer

Cell proliferation, inhibition of apoptosis, and acquisition of invasive capacity are hall marks of tumour development and progression. These processes imply major functional and morphological changes which occur at the cell membrane. Physical and functional interaction between membrane proteins and phospholipids affects survival, cell adhesion and, consequently, migration and invasion.

Lipids constitute at least 50% of the total cellular membrane and exert a myriad of functions as signaling and structural molecules .

Changes in lipid environment regulate function and availability of intrinsic membrane proteins by influence on membrane fluidity and cell polarization; thereby affecting inside out and outside in cell signaling .

Altered cellular lipid metabolism contributes to the onset of cancer and its progression . Phosphatidylinositol 3- phosphate (3-PIPs) mediates survival and migratory signals; and its inactivation by phosphatase and tensin homologue (PTEN) is associated to cancer progression, including breast cancer . Changes in sphingolipid metabolism may confer survival advantages to cancer cells . In addition, membrane signalling that leads to apoptosis, survival and migration is modulated by the relative ceramide / sphingomyelin (SM) / cholesterol levels in lipid rafts and may influence cell transformation and tumour progression . Therefore, changes in the lipidome of cancer cells reflect metabolic alterations associated to cancer development and progression.

Doria et al. studied the alterations of phospholipid profiles in the onset and evolution of cancer, in particular lipidomic analysis has been extended to human mammary epithelial cells and breast cancer cell lines. The most abundant PL class in all cell lines was PC, followed by PE, PS, cardiolipin (CL), PI, and SM; LPC was the less abundant PL found.

Differences in phosphatidylethanolamine (PE) content relative to total amount of PLs was highest in non-malignant cells while phosphatidic acid (PA) was present with highest relative abundance in metastatic cells. PI, PC, LPC, SM and PS relative abundance did not vary between cell types.

Types Relative CL amounts did not significantly vary between cell lines but alterations in the CL content, acyl chain composition and peroxidation in apoptotic and neurodegenerative diseases has been described .

Therefore, CL molecular species may be altered in breast cancer.

6.3 The Fluidity of Membranes

Membranes are not static sheets of molecules locked rigidly in place. A membrane is held together primarily by hydrophobic interactions, which are much weaker than covalent bonds. Most of the lipids and some of the proteins can shift about laterally—that is, in the plane of the membrane, like partygoers elbowing their way through a crowded room **(Fig. 16)**. It is quite rare, however, for a molecule to flip-flop transversely across the membrane, switching from one phospholipid layer to the other; to do so, the hydrophilic part of the molecule must cross the hydrophobic interior of the membrane.

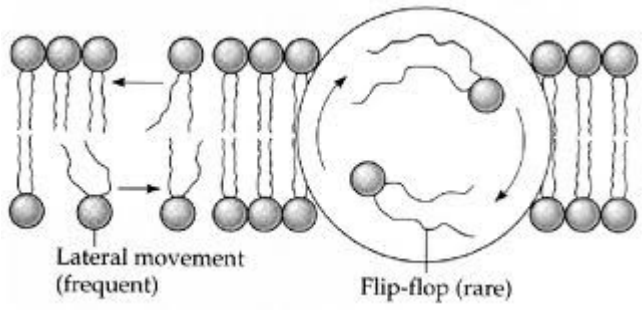


Figure 16 Movement of phospholipid

The lateral movement of phospholipids within the membrane is rapid. Adjacent phospholipids switch positions about 107 times per second, which means that a phospholipid can travel about 2 μm—the length of many bacterial cells—in 1 second. Proteins are much larger than lipids and move more slowly, but some membrane proteins do drift. And some membrane proteins seem to move in a highly directed manner, perhaps driven along cytoskeletal fibers by motor proteins connected to the membrane proteins' cytoplasmic regions. However, many other membrane proteins seem to be held immobile by their attachment to the cytoskeleton or to the extracellular matrix. The membrane remains fluid to a lower temperature if it is rich in phospholipids with unsaturated hydrocarbon tails. Because of kinks in the tails where double bonds are located, unsaturated hydrocarbon tails cannot pack together as closely as saturated hydrocarbon tails, and this makes the membrane more fluid . The steroid cholesterol, which is wedged between phospholipid molecules in the plasma membranes of animal cells, has different effects on membrane fluidity at different temperatures. At relatively high temperatures—at 37°C, the body temperature of humans, for example—cholesterol makes the membrane less fluid by restraining phospholipid movement. However, because cholesterol also hinders the close packing of phospholipids, it lowers the temperature

required for the membrane to solidify. Thus, cholesterol can be thought of as a "fluidity buffer" for the membrane, resisting changes in membrane fluidity that can be caused by changes in temperature. Membranes must be fluid to work properly; they are usually about as fluid as salad oil. When a membrane solidifies, its permeability changes, and enzymatic proteins in the membrane may become inactive if their activity requires them to be able to move within the membrane. However, membranes that are too fluid cannot support protein function either. Therefore, extreme environments pose a challenge for life, resulting in evolutionary adaptations that include differences in membrane lipid composition.

The fluidity of the lipid bilayer depends on the temperature and properties of the composing lipids. In the "fluid" liquid crystalline phase ($L\alpha$), the fatty acid chains are in a relatively flexible and disordered state. If the fatty acid chains are in a rigid and ordered state, the lipid bilayer is in a "gel phase" ($L\beta$). The transition from gel phase ($L\beta$) to the fluid phase ($L\alpha$) occurs abruptly at a characteristic temperature, Tm, called the "phase transition temperature" of lipid (**Fig. 17**). Generally, for lipids with same head groups but different lengths of saturated fatty acid chains, those with longer chains tend to have higher Tm. For example, 1,2-Dilauroyl-*sn*-Glycero-3-Phosphocholine (DLPC) with 12 carbon atoms in the fatty acid chain has its Tm at -1ºC; while for 1,2-Distearoyl-*sn*-Glycero-3- Phosphocholine (DSPC), with 18 carbon atoms, the Tm increases to 55ºC. Also, for lipids with same length of fatty acid chains, introduction of one or two carbon-carbon double bonds (CH=CH), especially those of *cis*- configuration, into the chain makes the chains more disordered and greatly decreases the phase transition temperature Tm. For example, 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC), with the same chain length as DSPC but one CH=CH bond in *cis*-configuration in each chain, has a Tm of -20ºC, well below that of DSPC .

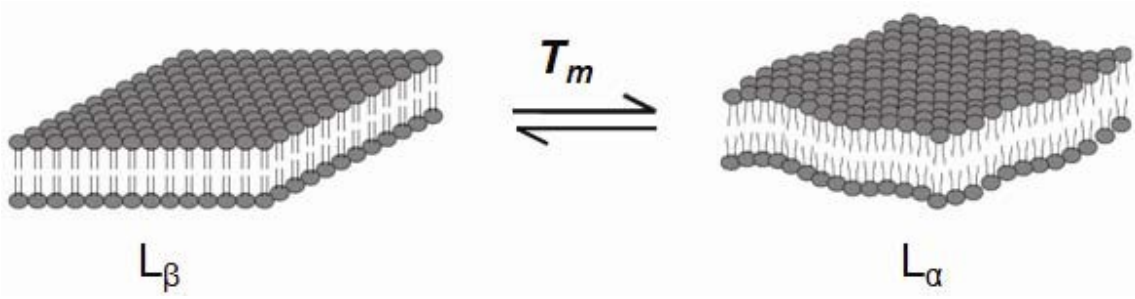


Figure 17 Schematic representation of gel-liquid phase transition of lipid membrane

For some membrane lipids, such as phosphatidylcholines, the lipid disordering occurs in two steps when increasing temperature. A first transition is observed a few degrees below the main transition T_m. This pretransition may be due to changes in the vicinity of the polar head group such an increase of the interaction of the lipid head groups with the solvent (Heimburg, 2000). For example, phosphatidylethanolamines that differ from phosphatidylcholines by the nature of the polar head group do not display a pretransition (McIntosh, 1980). According to Heimburg , pretransition and main transition are both part of the chain melting transition with the splitting into two transitions being the consequence of simultaneous changes in the lipid order and membrane curvature. Consequently, for the lipids that exhibit a pretransition temperature, an additional lamellar phase exists. This phase, called the ripple phase (P_β), is characterized by periodic one-dimensional undulations on the surface of the lipid bilayer . As this phase appears prior to the main chain melting, it must correspond to a partially disordered lipid phase. For this reason, it has been supposed that the undulations observed on the top of the lipid bilayers arise from periodic arrangements of linear ordered and disordered lipid domains . In presence of cholesterol, lipid bilayers can adopt an extra lamellar phase, called the liquid-ordered phase, which shares the characteristics of both gel and fluid phases . In other words, this phase resembles to the gel phase with less lateral packing order and at the same time to the fluid phase with more packing order. The incorporation of cholesterol into a solid-ordered lamellar phase disturbs the lateral triangular lattice and consequently reduces the ordering of the lipid chains. At the opposite, in a liquid-disordered lamellar phase, the rigid hydrophobic moiety of cholesterol is intercalated between the lipid chains and favors a *trans* chain conformation .Consequently, the liquid-ordered phase displays both a lateral and a rotational diffusion that are close to the ones of the liquid-disordered phase, but a conformational order similar to the one of the solid-ordered phase .

6.4 Lipidic polymorphism

In aqueous medium, lipid bilayer constituting the biological membranes can exist in different physical states, which are characterized by the lateral organization, the molecular order as well as the mobility of the lipid molecules within the bilayer. Consequently, physicochemical parameters such as temperature, pH, ionic strength and other factors such as the chemical structure of the lipid constituents and the presence of cholesterol strongly influence the nature of the lamellar phase. The molecular shape of a lipid can induce a specific assembly: this can be in the form of spheres of lipid molecules (micelles), pairs of layers that face one another (lamellar phase, observed in biological

system as a lipid bilayer), a tubular arrangement (hexagonal), or various cubic phases the cone-shaped lipids form inverted hexagonal structures, the cylindrical shaped ones form bilayers and the inverted cone-shaped lipids form micelles (**Fig. 18**).

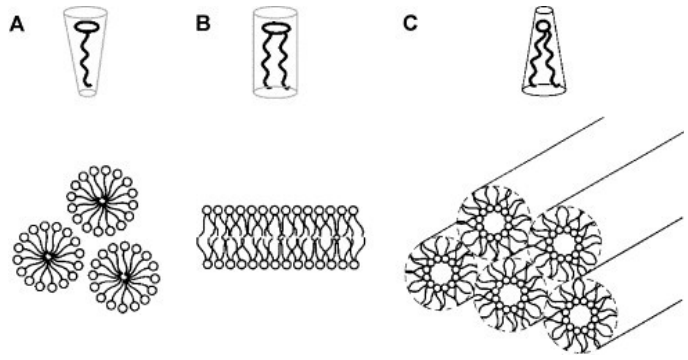


Figure 18 Phospholipid assemblies: bilayer (left top), micellar (left bottom) and inverted hexagonal (right) structures.

It is remarkable that the large majority of the membrane lipids will adopt only two types of structure in isolation, the lamellar or the hexagonal H_{II} phase. Micellar phospholipid organizations have only been observed for minority membrane lipids such as gangliosides and lysophospholipids. The quantitatively most important type of H_{II} lipids are PE and monoglucosyl- and monogalactosyldiglycerides . However, biological membranes also include significant amounts of lipids that are prone to exist in a gel phase at the physiological temperature (e.g. DPPC) or in a non-bilayer phase (e.g. cone shaped lipids). The non-bilayer forming lipids are able to produce stress in the 18 bilayer plane due to their cone shaped structure and to form a so-called frustrated state. This has been suggested to be important as a regulator of various functions, such as membrane fusion and membrane-protein interaction . The phase behavior of phospholipid membranes is strongly affected by the cholesterol, which is, together with phospholipids, a major lipid component of eucariotic membranes . The incorporation of cholesterol broadens the cooperative phase transition from a gel to a liquid-crystalline state, i.e. the transition is not sharp but takes place over a broader temperature range. At high concentrations, cholesterol even eliminates this transition. Cholesterol is able to induce a liquid-ordered phase where the orientational order of the hydrocarbon chains is increased . Mostly due to that, the surface area per phospholipid is decreased. The OH group of cholesterol has been suggested to reside at the height of ester carbonyl oxygens of PCs but not to form any tight complex with PC molecules. Also, due to tighter packing of the hydrocarbon chains, the passive permeability across the membrane decreases in the presence of cholesterol. The tight packing of PC and cholesterol molecules has been suggested to lead to the formation of stoichiometry-dependent superlattices . Interestingly, cholesterol

molecules have been found to favor interaction with SM as compared to PC . SM is, on average, more saturated than PC. It has been suggested that this enhances hydrophobic interactions between the aliphatic chains of SM and the rings of cholesterol. Also the possibility of enhanced hydrogen bonding has been proposed. All biological membranes include a variety of both integral and peripheral proteins, which are the main functional units of membranes. However, the lipid composition of biological membranes varies, depending on the functional properties of a membrane, and induced changes in the lipid composition are known to disturb or even damage the membrane functions . Thus, the properties of the lipids, such as the effective molecular shape, are prone to dramatically affect the function of the membrane proteins. There is evidence suggesting the existence of two-dimensional structures such as domains and superstructures in membranes consisting of a mixture of lipids . These structures may form due to preference of interactions or repulsion between lipid species. An example of this is the lateral segregation of lipids due to different chain length in a so called hydrophobic mismatch . The properties of the domains vary due to different composition, and even different phases may exist. The domains could be important in the regulation of the function of membrane proteins and also of the enzymes interacting with membranes .Also, the existence of variability in the lipid lateral organisation is prone to be important in complicated bilayer systems such as the endoplasmic reticulum and Golgi apparatus, in which high local curvature of the membrane is observed. In addition to bilayer structures, pulmonary surfactant monolayers at the water-air interface as well as lipoprotein particles are examples of biologically relevant non-bilayer lipid assemblies. Spherically shaped lipoprotein particles have a surface monolayer consisting of a mixture of phospholipids, cholesterol, and protein. The separation of hydrophobic lipids of triacylglyceride and cholesteryl ester molecules from the aqueous environment of blood plasma is stabilised by the surface monolayer.

Research's Aim

Olive oil has compounds that provide health benefits, including the prevention and treatment of diseases. Among olive oil compounds, the phenolic fraction has received considerable attention in recent years. Evidence from several studies have revealed that the protective effects of EVOO against chronic diseases such as atherosclerosis, cancer, obesity, diabetes, and coronary diseases are related to the phenolic compounds.

The bioactivity of the phenolic compounds in these chronic diseases could be related to different properties such as antioxidant and anti-inflamatory, although the molecular mechanism of these compounds in relation to many diseases could have different cellular targets.

The most important phenolic compounds that have been identified on EVOO may be divided into different groups such as phenolic acids, phenolic alcohols, secoiridoids, lignans, and flavones.

Most common problems are poor absorption, low half-life, due to rapid clearance and inactivation by metabolic enzymes. The development of suitable drugdelivery systems and, in particular, of liposomes, may be an appropriate strategy for the effective administration and of these compounds.

Liposomes increase drugs' half life and reduce their clearance, but they don’t discriminate among the different cellular types, then this is a factor limiting their effectiveness and suitability to be used in cancer therapy.

For this reason, in the last decade the research against cancer is oriented towards 'targeted therapy, which is based on the possibility to deliver drugs to specific tumor sites, narrowing the field of action compared to conventional chemotherapy and decreasing side effects. The specific aims were:

1. To develop liposomal formulations of EVOO 7 and EVOO 12 in order to improve their bioavalability and their cytotoxic effects on human breast cancer cellular models.
2. To design immunoliposomes containing the anti-HER2 antibody to target breast cancer cells expressing that surface antigen;
3. To evaluate the cytotoxic effects of the compounds in three different systems: free compounds, compounds incorporated into regular liposomes and incorporated into anti-HER2 immunoliposomes on two breast cancer cell lines showing different HER2 expression levels;
4. To study the effect of pinoresinol, a lignan compound, on phospholipid model membranes to evaluate the membrane-drug interaction.

Materials and Methods

1. Olive Oil

The olive oils used in this study were from five different monovarietal EVOOs obtained from different geographic zones in Spain: two Hojiblanca olive oils produced in Malaga (EVOO 1) and Sevilla (EVOO 9), seven Picual olive oils produced in Malaga (EVOO 2), Jaen (EVOOs 4, 10 and 11), Granada (EVOOs 5, and 6), and Cordoba (7), one Cornezuelo (EVOO 3), one Manzanilla (EVOO 8), and three Arbequina olive oils (EVOOs 12, 13, and 14).

The EVOOs were produced in the same year (September 2008). Olives were processed by continuous industrial plants equipped with a hammer crusher, a horizontal malaxator, and a two-phase decanter. Samples were stored in bottles without headspace at room temperature and darkness before analysis. To isolate the phenolic fraction of olive oils from all varieties, solid phase extraction (SPE) with Diol-cartridges was used. EVOO (60 g) was dissolved and loaded onto the column. The cartridge was washed with 15mL of hexane, which were then discarded in order to remove the non polar fraction of the oil. Finally, the sample was recovered by passing through 40 mL of methanol and the solvent was evaporated under vacuum. The residue was dissolved with 2 mL of methanol and filtered through a 0.25 μm filter before the RRLC analysis. The extracts of olive oils were diluted (1:10, v: v) with methanol.

2. Breast Cancer Cell Lines and Culture Conditions

We compared the anti-proliferative effects of EVOO phenolic extracts using two breast cancer cell models: MCF-7 breast cancer cells, which express physiological levels of HER2 (*i.e.* one single copy of HER2 gene) and JIMT1, which overexpress HER2.

JIMT1 cell line was derived from a pleural metastasis of a 62-year old patient with breast cancer who was clinically resistant to trastuzumab. It's a relatively novel cell line, commercially available since 2004. JIMT-1 cells grow as an adherent monolayer and form xenograft tumors in nude mice. As a cell line, JIMT1 carry phenotypic hallmarks of HER-2-positive breast cancer that is, histologically, representing a high-grade invasive ductal carcinoma lacking expression of estrogen and progesterone receptors . Although it is a HER2 positive cell line, it is resistant to the conventional therapy with trastuzumab .

MCF-7 is a cell line that was first isolated in 1970 from the breast tissue of a 69-year old Caucasian woman. MCF-7 cells are useful for in vitro breast cancer studies because the cell line has retained several ideal characteristics particular to the mammary epithelium.

These include the ability for MCF-7 cells to process estrogen, in the form of estradiol, via estrogen receptors in the cell cytoplasm. This makes the MCF-7 cell line an estrogen receptor (ER) positive control cell line. In addition to retaining their estrogen sensitivity, MCF-7 cells are also sensitive to cytokeratin. They are unreceptive to desmin, endothelin, GAP, and vimentin. When grown in vitro, the cell line is capable of forming domes and the epithelial like cells grow in monolayers. Growth can be inhibited using tumor necrosis factor alpha (TNF alpha), and treatment of MCF-7 cancer cells with anti-estrogens can modulate insulin-like growth factor finding protein's, which ultimately have the effect of a reduction in cell growth.

The main difference between both cell lines is that, differently to JIMT1, MCF7 cells present a very low HER2 expression.

These cellular line were routinely grown in Dulbecco's modified Eagle medium (DMEM) 50 units/mL penicillin, 50 mg/mL streptomycin (GIBCO), and 10% of heat-inactivated fetal bovine serum (FBS) (GIBCO).

Cells were maintained at 37 ◦C in a humidified atmosphere of 95% air and 5% CO_2.

2.1 Thawing of JIMT1 and MCF7 cells

Cells were kept in liquid nitrogen in cryotubes. Tubes were removed from the liquid nitrogen tank and put into a 37°C water bath for about 30-60 sec. The cells were transferred to a 15 mL tube using 5 mL pre-heated DMEM and centrifuged at 1500 rpm for 5 min. Media was removed by aspiration and the cell pellet was "flipped" and resuspended in 5 mL DMEM before transferred to a T25 flask.

2.2 Passaging of JIMT1 and MCF7 cells

Cells were passaged every 3rd or 4th day, when they had reached 100% confluence. Media was removed by aspiration using sterile Pasteur pipette and a vacuum line. 5 mL phosphate buffered saline (PBS) was added twice to the opposite side of where the cells were attached. The flask was gently rocked about 5 times to wash away media residues from the cells. PBS was removed by aspiration. 1 mL Trypsin/EDTA (Ethylenediaminetetraacetic acid) (GIBCO) (in case of T25 flask use), or 2 mL Trypsin/EDTA (in case of T75 flask use) was added directly to the cells and the flask was incubated for 5 min in 37°C, 5% CO_2. Cells were detached by gently tapping the flask. To neutralize trypsin's effect an amount of DMEM five times more of total trypsin volume

was added to the flask; that is 5 mL of DMEM for 1 mL of trypsin/EDTA or 10 mL of DMEM for 2 mL of trypsin/EDTA. Cells were suspended in the media, transferred to a 15 mL tube and centrifuged at 1500 rpm for 5 min. Supernatant was removed by aspiration and the cell pellet was "flipped" to separate the cells. The cells were resuspended in 10 mL DMEM and passaged into T75 flasks containing 5 mL DMEM yet.

3. Metabolic Status Assessment (MTT-Based Cell Viability Assays)

The ability of EVOO phenolic compounds to affect breast cancer cell viability was determined using a standard colorimetric MTT (3–4, 5-dimethylthiazol-2-yl-2, 5-diphenyl-tetrazolium bromide) reduction assay.

Traditionally, the determination of cell growth is done by counting viable cells after staining with a vital dye. Several approaches have been used in the past. Trypan blue staining is a simple way to evaluate cell membrane integrity (and thus assume cell proliferation or death) but the method is not sensitive and cannot be adapted for high throughput screening. Measuring the uptake of radioactive substances, usually tritium-labeled thymidine, is accurate but it is also time-consuming and involves handling of radioactive substances.

Solutions of MTT solubilized in tissue culture media or balanced salt solutions, without phenol red, are yellowish in color. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals which are insoluble in aqueous solutions (**Fig.19**). The crystals can be dissolved in a organic solvent such as Dimethylsulfoxide (DMSO) or isopropanol and the released, solubilised formazan reagent is spectrophotometrically measured at 570 nm. The assumed advantage of this cell assay system is that this reaction can only take place in living cells with functional mitochondria. It is also assumed that the amount of formazan formed during a given exposure period is directly proportional in the number of viable cells per well. The resulting purple solution is spectrophotometrically measured. An increase in cell number result in an increase in the amount of MTT formazan formed and an increase in absorbance.

When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-response curve.

The MTT method is useful in the measurement of cell growth in response to mitogens, antigenic stimuli, growth factors, cytotoxicity studies, so it's possible derivate cell growth curves.

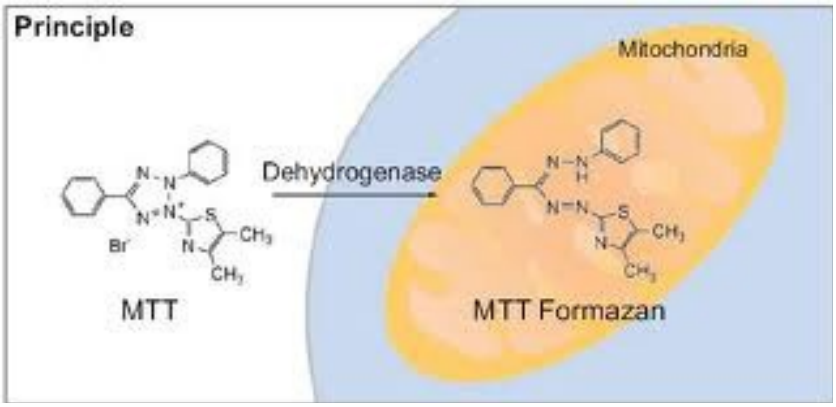


Figure 19 Cleavage of the yellow-colored tetrazolium salt, 3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) into a purple-colored formazan by the mitochondrial enzyme succinatedehydrogenase

3.1 Experimental procedure

Cell suspensions were seeded into 96-well microplates. They were left one day in order to reach a confluence of 80%. Then medium were aspirated, cells were treated with different concentrations of the whole crude EVOO-PE dissolved in ethanol and the plates were incubated at 37°C for 72 hours. Ethanolic dilutions were prepared immediately before starting each experiment by diluting 100% full strength EVOO-PE (i.e., 1 mL of the methanol extract was evaporated under vacuum to give the dried methanol extract; after a complete solvent removal, dried methanol extract was dissolved in 95% ethanol) in fresh culture medium. An appropriate amount of ethanol (v/v) was added to control cells.

After 3 days, medium and consequently drugs were aspirated and MTT was added (100 µL of a solution of 5% MTT in PBS). After 3-5 hours of incubation in 37°C in dark, MTT was removed by aspiration and 100 µL of DMSO were added in each well; to allow the solubilisation of formazan's crystal, plates were mixing on an agitator for 10 minutes and cellular viability was quantified using an absorbance spectrophotometer microplate reader (SPECTRO starOmega, BMG LABTECH) measuring absorbance at 570 nm with background correction at 620 nm. The absorbance measured was proportional to the number of living cells in each well. The cytotoxic IC50 values (inhibitory concentration 50%) for the drugs were determined from log concentration-effect curves in GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA), using a variable slope sigmoidal

curve fit to experimental data. Data are presented as the means ± standard error of the mean.

4. Quantification and identification of EVOO polyphenols compounds

4.1 RRLC-MS Analysis

The usual technique to analyze the phenolic fraction is high performance liquid chromatography (HPLC) . High performance liquid chromatography (HPLC) is a technique used to separate a mixture of compounds with the purpose of identifying, quantifying and purifying the individual components of the mixture. The sample to be separated and analyzed is introduced, in a discrete small volume, into the stream of mobile phase percolating through the column. The components of the sample move through the column at different velocities, which are functions of specific physical or chemical interactions with the stationary phase. The velocity of each component depends on its chemical nature, on the nature of the stationary phase (column) and on the composition of the mobile phase. The time at which a specific analyte elutes (emerges from the column) is called the retention time. The retention time measured under particular conditions is considered an identifying characteristic of a given analyte. The stationary phase may be a porous solid, usually in a thin layer of particles or a substance bound to a solid support. The mobile phase designates the liquid (a solvent or a mixture of solvents) flowing over the particles.

A HPLC system is basically composed of 1) a pump, 2) an injector, 3) a column, 4) a detector and 5) data acquisition system (**Fig. 20**).

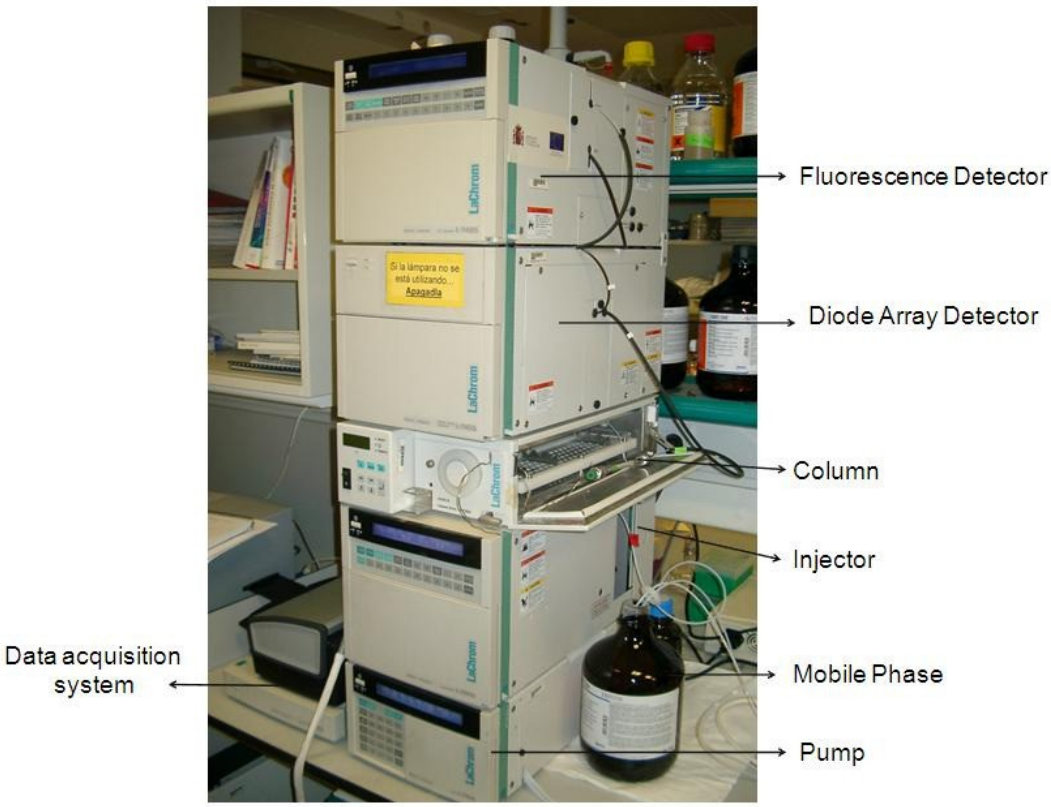


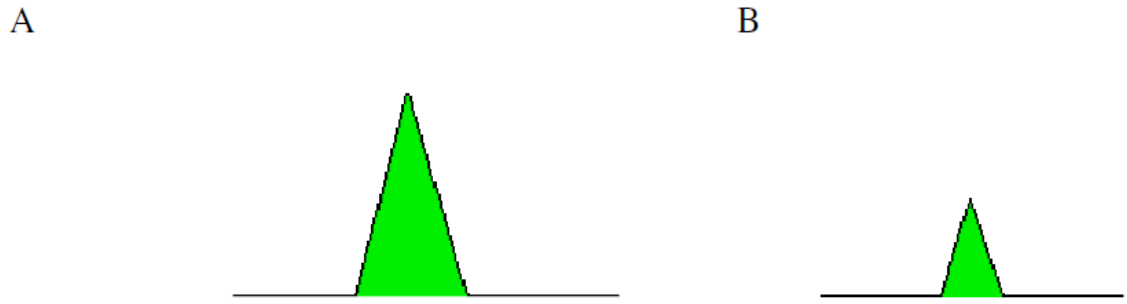
Figure 20 HPLC components

The column is a stainless steel tube which is packed with spherical solid particles of stationary phase. Mobile phase is constantly fed into the column inlet at a constant rate by a liquid pump. The sample is injected from a sample injector, located near the column inlet. The injected sample enters in the column with the mobile phase and the components in the sample migrate through it. Compounds that tend to be affine to the mobile phase therefore migrate faster through the column while compounds that tend to be affine to the stationary phase migrate slower. If the sample it is not a pure compound, but an mixture, as the EVOO polyphenolic extracts, its components will distribute between the two phases separately in so that those who have more affinity with the mobile phase, are not retained by the stationary phase and elute for first. In opposition to those with higher affinity for the stationary phase will advance more slowly through the column and elute later. In this way, each component is separated on the column and sequentially elutes from the outlet. Each compound eluting from the column is detected by a detector connected to the outlet of the column. When the separation process is monitored by a recorder starting at the time the sample is injected, it's possible to obtain a graph. This graph is called a chromatogram. The time required for a compound to elute (called retention time) and the relationship between compound concentration (amount) and peak area depend on the characteristics of the compound. Retention time is therefore used as an index for qualitative determination and

area under curve (AUC) as an index for quantitative determination. In this sense, the smaller the AUC, the less concentrated the compound is, as shown in the simplified diagram in **Fig. 21 A** and **B**.

The retention time of the target compounds and the concentration for each unit of peak area are based on data obtained by analyzing samples with known quantities of the reference standards. Normally, the reference standards are highly purified target compounds.

Figure 21 Schematic diagrams of preparation at different concentrations. (A) At left the more concentrated,



(B) at right the less concentrated

Recently, an improvement in chromatographic performance has been achieved by the introduction of rapid-resolution LC (RRLC) and ultraperformance LC (UPLC). These approaches use narrow bore columns packed with very small particles (1.8 μm) and high flow rate with delivery systems operating at high backpressures. The major advantages of RRLC and UPLC over conventional HPLC are improved resolution, shorter retention times, higher sensitivity, and better performance. Coupling RRLC with mass spectrometry (MS) further offers a potent analytical alternative, which has been applied in recent publications characterizing food products.

4.1.1 Experimental protocol

The development of a rapid resolution liquid chromatography (RRLC) coupled to electrospray time-of-flight mass spectrometry (ESI-TOF-MS) method to characterize the phenolic profile in EVOOs was performed in an Agilent 1200-RRLC system (Agilent Technologies, Waldbronn, Germany) of the Series Rapid Resolution equipped with a vacuum degasser, autosampler, a binary pump, and a UV-vis detector. The chromatographic separation was carried out on a Zorbax Eclipse Plus C18 analytical column (4.6 mm × 150 mm, 1.8 μm particle size). The flow rate was 0.80 mL/min, and the temperature of the column was maintained at 25 ° C. The mobile phases used were water with 0.25% acetic acid as eluent A and methanol as eluent B. The optimal chromatographic method consisted in the following multistep linear gradient:

0 min, 5%B; 7 min, 35%B; 12min, 45% B; 17 min, 50% B; 22 min, 60% B; 25 min, 95% B, 27 min, 5%B, and finally a conditioning cycle of 5 min with the same conditions for the next analysis. The injection volume in the RRLC was 10 μL. The compounds separated were monitored in sequence first with DAD (240 and 280 nm) and then with a mass spectrometry detector. MS was performed using the microTOF (Bruker Daltonik, Bremen, Germany) which was coupled to the RRLC system. At this stage, the use of a splitter was required to the coupling with the MS detector as the flow which arrived to the TOF detector had to be 0.2mL/min in order to obtain reproducible results and stable spray. The TOF mass spectrometer was equipped with an ESI interface (model G1607A from Agilent Technologies, Palo Alto, CA) operating in negative ion mode. External mass spectrometer calibration was performed with sodium formiate clusters (5 mM sodium hydroxide in water/2-propanol 1/1 (v/v), with 0.2% of formic) in quadratic + high precision calibration (HPC) regression mode. The calibration solution was injected at the beginning of the run, and all the spectra were calibrated prior to polyphenol identification. The optimum values of source parameters were: capillary voltage of +4 kV; drying gas temperature, 190 °C; drying gas flow, 9 L/min; nebulizing gas pressure, 2 bar, and end plate offset, -0.5 kV. The values of transfer parameters were: capillary exit, -120 V; skimmer 1, -40 V; hexapole 1, -23 V, RF hexapole, 50 Vpp, and skimmer 2, -22.5 V. The source and transfer parameters were get for a good sensitivity and reasonable resolution of the mass range for compounds of interest (50-1000 m/z) in order to improve ionization performance.

The accurate mass data for the molecular ions were processed using the software Data Analysis 3.4 (Bruker Daltonik), which provided with a list of possible elemental formulas by using the Generate Molecular Formula Editor. The latter uses a CHNO algorithm

providing standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double bonds equivalent, as well as a sophisticated comparison of the theoretical with the measured isotopic pattern (Sigma-Value) for increased confidence in the suggested molecular formula. The widely accepted accuracy threshold for confirmation of elemental compositions has been established at 5 ppm for most of the compounds.

4.2. Determination of total phenols by Folin-Ciocalteu technique

Total phenol content of different EVOO extracts was determined using Folin-Ciocalteu technique.

The Folin –Ciocalteu reagent or Folin's phenol reagent or Folin-Denis reagent, also called the Gallic Acid Equivalence method (GAE), is a mixture of phosphomolybdate ($H_3PMo_{12}O_{40}$) and phosphotungstate ($H_3PW_{12}O_{40}$) used for the colorimetric assay of phenolic and polyphenolic antioxidants. These are reduced by the action of phenols in a mixture of tungsten (W_8O_{23}) and molybdenum (Mo_8O_{23}), (blue oxides). The blue color produces a maximum absorbance at 700-750 nm. The coloration obtained can be measured at 700 nm and this absorbance is proportional to the total amount of phenolic groups in the samples.

4.2.1 Experimental protocol

The total phenolic concentration was determined according to the Folin-Ciocalteu method using gallic acid as the standard.

50µL EVOOs' extracts was assayed with 250 µL of Folin reagent and 500 µL of saturated solution of sodium carbonate.

The mixture was diluted with water to a final volume of 5 mL. The absorbance relative to that of the blank was measured using Spectronic Genesys 5 (Rochester**,** NY, USA) at 700 nm after incubation for 1 h at room temperature. A calibration curve with different concentrations of gallic acid was prepared (0, 50, 100, 150, 250, and 500 mg/L). The data for the total phenolic content were expressed as gallic acid equivalents (mg/100 ml).

To quantify the phenolic concentration in liposome or immunoliposome SDS 10% was added to mixture for liposomes and immunoliposome's break. All the sample's measurements were made in triplicate.

5. Protein extraction and analysis

5.1 Preparation of cell lysates

Protein extracts were made from cells when approximately 80% confluent. Cells were washed twice with PBS and were scraped with a cell scraper, the lysate were transferred to a 1.5 ml tube and centrifuged at 1500 rpm for 5 min. The cellular pellets were lysed in a lysis buffer containing 50 mM Tris pH 7.4, 1% Igepal CA-630, 150 mM NaCl, 5 mM EDTA and 10 mg/mL of protease inhibitor cocktail (Sigma–Aldrich, Europe). Cells were kept on ice for 20 min and, after a freezing/thawing cycle, lysates were centrifuged at 12,000 rpm for 5 min to remove particulate matter and cleared lysate was placed in a fresh 1.5 ml tube. Samples were then processed immediately, or frozen and stored at -20°C.

5.2 Determination of protein concentration

Protein concentrations were estimated using the Bradford colorimetric assay (Bio-Rad, Richmond, CA, USA). Protein-containing solutions were mixed with Bradford assay solution and the absorbance at 595 nm measured. The protein concentration was then determined based on a standard curve derived from protein standards of known concentration.

5.3 Separation of proteins by polyacrylamide gel electrophoresis (SDSPAGE)

Proteins for Western blot analysis were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The required amount of lysate to give 50 µg protein was mixed with 5 µl sample buffer and 2 µl sample reducing agent, and diluted to a total volume of 30 µl with lysis buffer. Samples were then heated at 95°C for five min, briefly centrifuged to collect the whole sample, and resolved on polyacrylamide gels by electrophoresis in gel tanks containing 1X running buffer (15 g Tris HCl pH 8.3, 72 g Glycine, 5 g SDS in a final volume of 1 L).

The gels were run at 120V for approximately 1 hr 30min. When the bromophenol blue dye front was seen to have reached the end of the gels, electrophoresis was stopped.

5.4 Western blotting

Following electrophoresis, the acrylamide gel was placed over the nitrocellulose membrane and sheets of pre-soaked filter paper were placed on top of the gel. Excess air was again removed by rolling a universal tube over the filter paper. The proteins were transferred from the gel to a nitrocellulose membrane at a current of 34mA at 15V for 2 hours.

The nitrocellulose membranes were blocked for 2 hours at room temperature with fresh 5% non-fat dried milk in Tris-buffered saline (TBS) with 0.5% Tween (Sigma–Aldrich, Europe). After blocking, the membranes was washed 3 x 5 min using 1X TBS/PBS. The membrane was then incubated with primary monoclonal anti-bodies against HER2 (sc-284, Santa Cruz Biotechnology, INC) for 1hr. The membrane was again was washed 3 x 5 min using 1X TBS/PBS and then incubated with horseradish peroxidase-linked secondary antibodies (Sigma–Aldrich, Europe). Finally the membrane was washed 3 x 5 min using 1X TBS/PBS. Bound antibody was detected using enhanced chemiluminescence (ECL) (Amersham International, Buckinghamshire, UK).

5.5 Enhanced chemiluminescence detection

Protein bands were developed using the Enhanced Chemiluminescence Kit (Amersham International, Buckinghamshire, UK) according to the manufacturer's instructions. The blot was removed to a darkroom for all subsequent manipulations. A sheet of parafilm was flattened over a smooth surface, e.g. a glass plate, making sure all air bubbles were removed. The membrane was placed on the parafilm, and excess fluid removed. 1.5 mls of ECL detection reagent 1 and 1.5 mls of reagent 2 were mixed and covered over the membrane. Charges on the parafilm ensured the fluid stayed on the membrane. The reagent was removed after one minute and the membrane covered in cling film. The membrane was exposed to autoradiographic film in an autoradiographic cassette for various times, depending on the signal. The autoradiographic film was then developed. Densitometric analyses were performed using the Sigma-Gel gel analysis software (Jandel Scientific, Chicago, IL, USA).

6. Flow cytometer analysis

6.1. Principle of FACS analysis

In a commun flow cytometer experiment, a cell suspension is injected through a nozzle whereby the cells are focused into the centre of a columnar flow by mechanical

properties of the fluid (hydrodynamics). The cells in the single-cell stream, passes through a light source usually generated from a laser beam or a mercury lamp. Each cell then scatters the laser light, which is recorded by differente detectors that give information about the size (forward scatter light, FSC) and granularity of the cell (side scatter light, SSC). If the cell that passes through the laser beam has been labelled with a fluorescent reagent (fluorochrome) it emits fluorescence and can be recorded by another detector. Fluorescence may also be emitted by the cells naturally (autofluorescence). Scattered and fluorescent light is collected by the optics, transferred through specific filters to photodetectors (phomultiplier tubes, PMTs) and further digitalised and analysed by a computer. Fluorochromes absorb light of certain specific colour and then emit light of a different colour, usually at longer wavelength. These fluorochromes may be coupled to antibodies, which are directed to molecules expressed on the cell surface or to intracellular markers. Thus, the fluorescence of a cell is proportional to the amount of molecules to which the antibody has bound.

Cells can be scanned at a high rate and a large number of cells can be enumerated and characterized individually upon their specificity, frequency, function, state of activation, differentiation, viability etc, which may give some information of the phenotype and function of different cell subsets.

In our research, flow cytometry has been used to verify that HER2 receptor, expressed in breast cancer cells, was present on cell surface.

6.2 Analysis of HER2 expression

MCF7 and JIMT1 were trypsinized and washed repeatedly with PBS EDTA 0.5% to obtain single cell suspensions. Then, one and a half million of cells of each cell line were fixed in 3.5% Formalin (Sigma–Aldrich Co., St. Louis, MO) during 5 minat 4 °C followed by 10 min at room temperature (RT). After three cycles of washing with PBS-EDTA 0.5%, cells were blocked for 30 min with PBS BSA 2% at RT. Once the blocking solution was removed, the cells were incubated overnight at 4 ºC with gently agitation with a rabbit anti-human HER2/neu antibody (sc-284, Santa Cruz Biotechnology, INC) at a concentration of 0.5 μg/105 cells. Cells were washed three times with PBS before proceeding with the secondary antibody staining. Anti-rabbit FITC-conjugated IgG (F-2765, Molecular Probes) was used as the secondary antibody at a dilution of 1:25 for 30 min at RT. As control, the secondary antibody alone was used after blockade to show nonspecific binding of the IgG

to cells. After washing and recollecting cells in PBS, the FITC-stained cells were quantitated by flow cytometry in an Epics XL instrument (Beckman Coulter Co., Miami, Florida) by analyzing the intensity of the green fluorescence associated to cells.

7. LIPOSOMES

7.1 Chemicals

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol) 2000] (D2000M) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Natural lipid egg yolk phosphatidylcholine (EYPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-PEG-2000 (DSPE-PEG) were obtained from Lipoid GmbH (Ludwigshafen, Germany). All lipids were dissolved in chloroform/methanol (1:1) and stored at -20°C. Herceptin TM (Trastuzumab) was obtained from ROCHE.

7.2 Liposomes preparation: thin film method

Two type of lipid composition were used in the present study, one containing only EYPC for simple liposome preparation and another one used for the immunoliposome preparation containing adequate amounts of each lipid combined in a molar ratio of 74.5:20:5:0.5 (EYPC:Chol:DSPE-PEG:D2000M), previous studies demonstrated that this was the optimal composition [195]. EVOO's extracts were added to the lipid mixture. The organic solvent was removed by evaporation under a stream of nitrogen (N_2) and a thin film of lipid was formed inside of the test tubes. The tubes were further vacuum-dried for 3-4 h to remove any residual organic solvent. Subsequently the lipid film was hydrated using Histidine buffer (THIS) (1.2g/L histidine–HCl, 0.78 g/L histidine, pH 7.4). The resultant liposomal suspension was agitated by vortexing at 37°C and a homogeneous milky suspension of MLVs was obtained.

7.3 Size Reduction of Liposomes

The liposomal suspension was filter-extruded through a 100 nm polycarbonate membrane Track-Etch Nuclepore membrane (Whatman, UK) in order to obtain large unilamellar vesicles (LUVs). Size reduction was done by 18 extrusion cycles by hand with a syringe extruder LiposofastTM (Avestin Inc., Canada) [196] or by LIPEXTM Extruder

(Northern Lipids Inc) for more concentrated preparations and bigger volumes unable to pass by liposofast (**Fig. 22)**.

The resultant products were stored at 4 °C until use.

A **B**

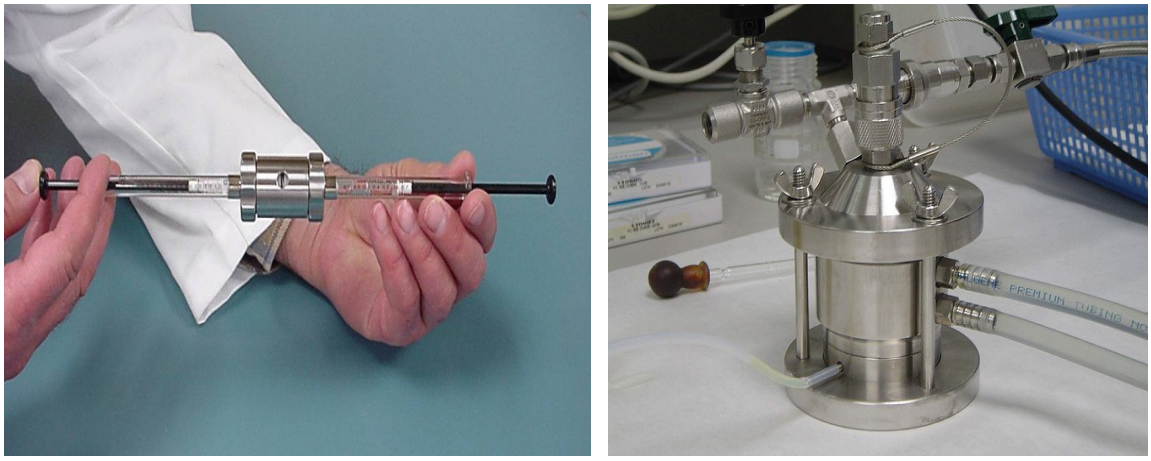


Figure 22 (A) Syringe extruder LiposofastTM (B) LIPEXTM Extruder

7.4 Immunoliposomes preparation: antibody derivatization and conjugation to liposomes

Chemical strategy for this immunoliposome preparation is based on:

1. Use of free amino groups present in the antibody molecules;
2. Modification of these functional groups by Traut's reagent (derivatization);
3. Use of a PEG derivate (D2000M) which acts as a linker between liposomes and antibodies.

Perham and Thomas [197] originally prepared an imidoester compound containing a thiol group, methyl 3-mercaptopropionimidate hydrochloride.

The imidoester group can react with amines to form a stable, charged linkage, while leaving a sulfhydryl group available for further coupling. Traut et al. [198] subsequently synthesized an analogous reagent containing one additional carbon, methyl 4-mercaptobutyrimidate. Later, this compound was found to cyclise as a result of the sulfhydryl group reacting with the intrachain

imidoester, forming 2-iminothiolane. The cyclic imidothioester still can react with primary amines in a ring-opening reaction that regenerates the free sulfhydryl. Traut's reagent is fully water-soluble and reacts with primary amines in the range of pH 7–10. The cyclic imidothioester is stable to hydrolysis at acid pH values, but its half-life in solution

decreases as the pH increases beyond neutrality [199]. **Fig. 23** describes the thiolation of antibody using Traut's reagent in the preparation of immunoliposomes.

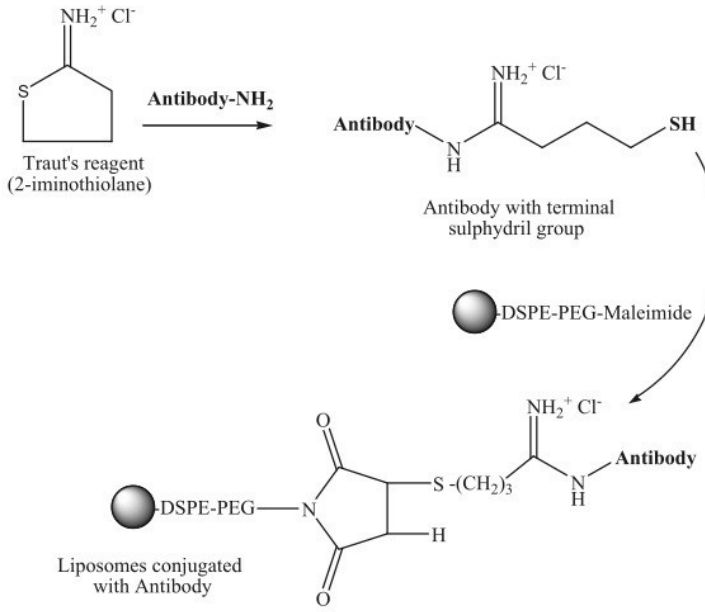


Figure 23 Thiolation of trastuzumab using Traut's reagent and conjugation of thiolated antibody to maleimide groups on liposome

7.4.1 Experimental protocol

Lipids were combined in a molar ratio of 74.5:20:5:0.5 and the EVOO extracts were added to mixture. Liposomes were formed by thin film method as described in the section 5.2; then liposomes'size was reduced in order to obtain LUVs. At the same time, trastuzumab was derivatized as previously described [195-200-201]. Briefly, the antibody was thiolated by reaction with 2-iminothiolane (Traut's reagent) for 2 hours and then incubated with the unilamellar vesicles in an Argon inert atmosphere for 12 h at room temperature with gentle agitation. During the reaction, the thiol group of the derivatized antibody reacted with the maleimide group of the D2000M lipid present in the liposome yielding the immunoliposome with the antibody covalently attached (the technique is thoroughly reviewed in [202]). After the derivatization, the unbound antibody was separated by size exclusion chromatography.

7.5 Separation of no-encapsulated compounds and unbound antibody from liposomes

7.5.1 Separation of no-encapsulated compounds from liposomes by Ultrafiltration

To separate no-encapsulated compounds from liposomes, ultrafiltration was used.

Ultrafiltration (UF) is the process of separating extremely small particles from fluids. Ultrafiltration can only separate molecules which differ by at least an order of magnitude in size.

Normally the membranes for the ultrafiltration are rated according to the nominal molecular weight limit (NMWL) or the molecular weight cut-off (MWCO). The membrane used for the experiments has a MWCO of 30,000; in this case free curcumin with a 368 MW, free resveratrol with 228 MW or both can be easily separated from encapsulated fraction.

In this study, the liposomal preparation was transferred to 15mL cellulose membrane ultrafiltration tubes (Amicon Ultra-15 Centrifugal Filters, Millipore,Europe). The samples were then centrifuged using Heraeus Biofuge PRIMO centrifugator (Heraeus, Europe). The centrifugation was done at speed of 1500 rpm for 15 minutes.

7.5.2 Separation of no-encapsulated compounds and unbound antibody from immunoliposomes by Size Exclusion Chromatography

Immunoliposome's formulation was added to a glass tube packed with Sepharose CL-4B (Sigma– Aldrich) to approximately 20 mL (in a glass column 20 cm height and 2 cm in diameter). THIS buffer was used for the elution and it was added repeatedly to insure continues flow and prevent the column from drying. The flow rate was estimated to be 2 mL/min. To detect when immunoliposome preparation passed through the column and collected it, the column was connected to an UV detector. Many organic compounds absorb UV light of various wavelengths; the passage of immonoliposomes and drugs through the detector results in a change of the signal.

7.6 Quantitative analysis of lipid concentration

Fiske's assay is a quantitative method to determinate phosphorus content and it is used to quantify the phospholipid solutions concentration. First, a standard solution of dipotassium hydrogen orthophosphate (K_2HPO_4) was prepared to a final concentration of 0.4 mM. Aliquots of phosphate stock solution were transferred to six glass tube in order to have a standard curve with 20, 40, 80, 120, 160 and 200 nmoles of phosphorus, respectively (**Fig. 24**). A volume of 0.4 mL of perchloric acid (HClO4) at 60% was added in the standard tubes and in the samples and incubated at 185°C for 30 minutes.

After cooling at room temperature, a volume of 4 mL of ammonium molybdate solution and 0.5 mL of ascorbic acid 10% were added and mixed. Standard tubes and samples were incubate into sand bath at 180-200°C for 15 minutes. Ammonium molybdate reacted with the acid producing a

coloured compound that can be measured spectrofometrically at 812 nm quantifying phosphorous content and indirectly lipid concentration. All the sample's measurements were made in triplicate.

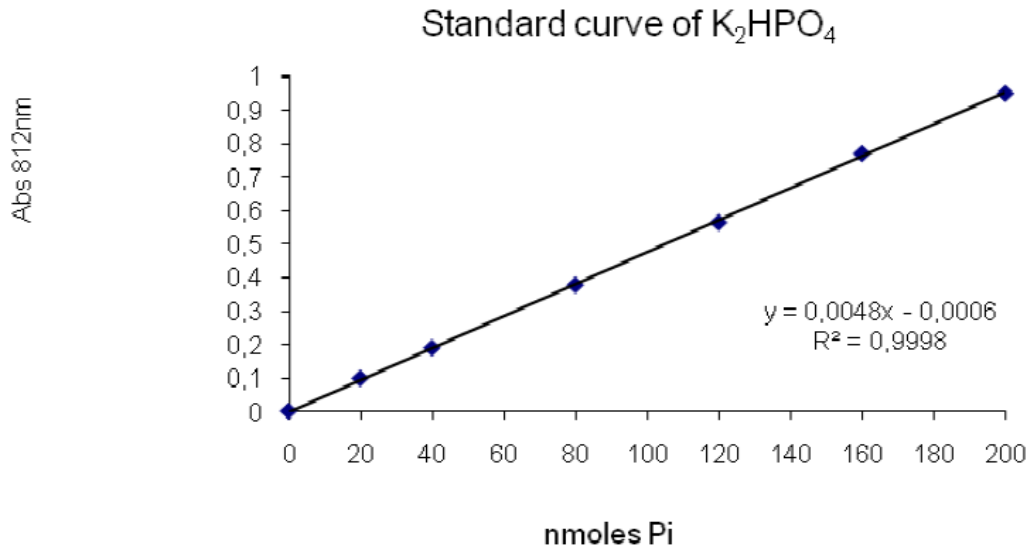


Figure 24 Standard curve of dipotassium hydrogen orthophosphate used for quantification of lipid content

7.7 Size determination of liposomes

Liposomes' s particle size was measured using the technique of dynamic light scattering (DLS) also known as photon correlation spectroscopy (PCS). The principle behind this technique is that when a light beam hits a particle which is in random motion or 'Brownian' motion, it causes a Doppler shift in the wavelength of the incoming light and this shift relates to the size of the particle. In this way, the larger is the particle the slower is the motion, while the smaller particles will move faster. This technique requires a very short time to measure the particle size.

Zetasizer Nano ZS (Malvern Instruments Ldt, UK) was used to measure the particle size of the liposomes. The particle size was measured using a He/Ne 4mW laser. As sample void liposomes and encapsulated liposomes were used.

Results were obtained from three series of measurements in which each measure has at least 12 replies. It was considered as accepted only the results that showed a polydispersity index (PDI) less than 0.2; PDI is a measure of samples' homogeneity, the more homogeneous sample is the lower the PDI is obtained.

The software of the instrument determinated the size of LUVs (Z, diameter in nm) and estimated the wideness of distribution (polyispersity index) in function of the intensity of the light scattered from particles.

8 Pinoresinol's effects on phospholipid model membrane

8.1 Chemicals

1,2-Dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DMPG), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dielaidoyl-*sn*-glycero-3 phosphoethanolamine (DEPE) were obtained from Avanti Polar Lipids (Birmingham, AL, USA).

Stock solutions of lipids were prepared in chloroform/ methanol (1:1) and stored at -20 ◦C. The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) were obtained from spectroscopic reagent grade. Double-distilled and deionized water was used throughout this work. Pinoresinol was obtained from Sigma-Aldrich (St. Louis, MO).

8.2 Determination of pinoresinol partition coefficient in model membranes (K_P)

The interaction with biological membranes may play an important role in order to explain the biological activity of Pinoresinol.

The partition coefficient of a chemical compound provides a thermodynamic measure of its hydrophilicity-lipophilicity balance. The partition coefficient between an aqueous phase (water or buffer) and an organic phase (n-octanol) is the most widely used measure of chemical compound lipophilicity. Higt lipophilicity often goes with poor acqueous solubility. This can bring with it many challenges often making development of a

seemingly promising drug candidate very difficult. Lipophilicity is also a major structural factor that influences the pharmacokinetic and pharmaco-dynamic behavior of compounds. Partitioning within a biological system and biological activity are governed by recognition forces that are, among others, defined by hydrophobic interactions. Strong hydrophobic interactions can result in unspecific binding with proteins (target and non-target) in the aqueous biological environment. A hydrophobic drug molecule has a thermodynamic tendency to reduce the surface area exposed to water. Hydrophobic compounds will tend to bind to hydrophobic surfaces through Van der Waals bonds. For compounds with extracellular or cell-surface targets it appears prudent to restrict lipophilicity and thereby avoid easy diffusion across biological membranes and into cells, cellular compartments, and the CNS. On the other hand, a certain degree of lipophilicity is required to allow a drug to enter cellular organelles or to cross the blood-brain barrier.

However, the ability of the octanol−water partition coefficient to describe drug partitioning has been questioned due to the major differences in the biophysical properties of octanol and phospholipid cell membrane. Due to this, alternative approaches, have been developed. The ability of pinoresinol to partition into phospholipid bilayers was tested through the determination of its phospholipid/water partition coefficient, Kp, using model membranes. This parameter has higher physiological significance than the regular octanol–water partition coefficient.

8.2.1 Spectroscopic measurements

We tried to determinate the partition coefficient of Pinoresinol using its spectroscopic characteristics.

Fluorescence spectra were recorded with a SLM-8000C spectrofluorimeter fitted with Glan-Thompson polarizers. All emission spectra were automatically corrected for instrument response at each wavelength. We tried to measure he partition coefficient (K_P) of Pinoresinol from its intrinsic fluorescence intensity increase upon the incorporation into large unilamellar vesicles (LUVs) composed of DMPC or DMPG as compared to that in the aqueous phase at 30 ◦C the appropriate pH value. Pinoresinol concentration was kept constant at 10 μM and phospholipid concentration was varied.

Samples were excited at 280 nm and fluorescence emission was recorded at 310 nm. MLVs were formed by resuspending the dried phospholipid in buffer (10mM HEPES, 0.1mMEDTA, 100mM NaCl, pH 7.4). In the case of DMPG the buffer pH was adjusted by

addition of HCl up to pH 3, and was controlled before and after the measurements. The vesicle suspension was then heated at a temperature above the phase transition of the phospholipid and vortexed several times. LUVs with a mean diameter of 100 nm were prepared from these MLVs by 18 extrusion cycles through 100 nm polycarbonate filters (Nucleopore, Cambridge, MA, USA) and phospholipid content was determined .The molar percentage of pinoresinol in all the experiments of the present study corresponds to the fraction of pinoresinol incorporated or associated to the phospholipid vesicles, determined from its phospholipid/water partition coefficient.

8.2.2 Determination of capacity factor k' $_{IAM}$ by IAM-HPLC

The impact of the lipophilicity of drug on their pharmacokinetic behavior and pharmacological activity has been recognized for a long time. In recent years the interest has focused on the importance aspect of drug-membrane interactions . As a consequence, membrane-like systems have been developed for the determination of partition coefficients, for instance liposomes, and high performance liquid chromatography (HPLC) methods. In 1989 a combination of an artificial lipid membrane and the fast analytical HPLC system was introduced .

Immobilized artificial membranes (IAMs) mimic the lipid environment of a fluid cell membrane on a solid matrix and are thus of particular interest for the prediction of drug partitioning into biological membranes.

The first IAM stationary phase was based on the prevalent membrane lipid, phosphatidylcholine (PC), and consists of monolayers of amphiphilic phospholipids covalently bonded to aminopropyl silica particles through a terminal amide linkage (**Fig.25**).

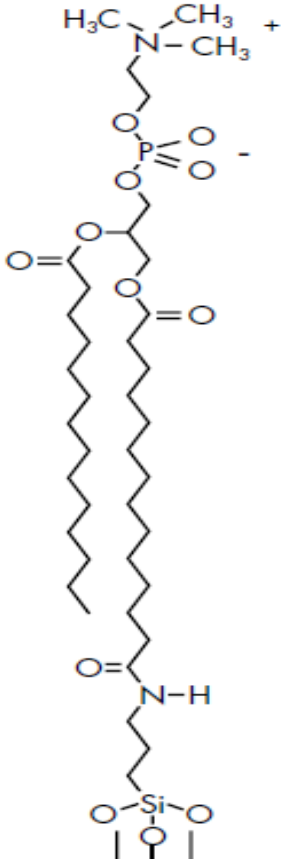


Figure 25 IAM PC.

IAM chromatography has recently gained acceptance among drug discovery chemists for estimating the membrane permeability of small molecule drugs.

Figure 26 illustrates that the interaction between membrane bilayer and drug can be modeled by the IAM column/drug system.

This IAM technique provides superior correlation with experimentally determined drug permeability when compared to other chromatographic methods. ODS silica, for example, retains analytes solely on the basis of hydrophobicity.

IAM more closely mimics the interaction of analytes with biological membranes, where a combination of hydrophobic, ion pairing, and hydrogen bonding interactions are possible.

This combination of interactions measured by the IAM column is known as phospholipophilicity .

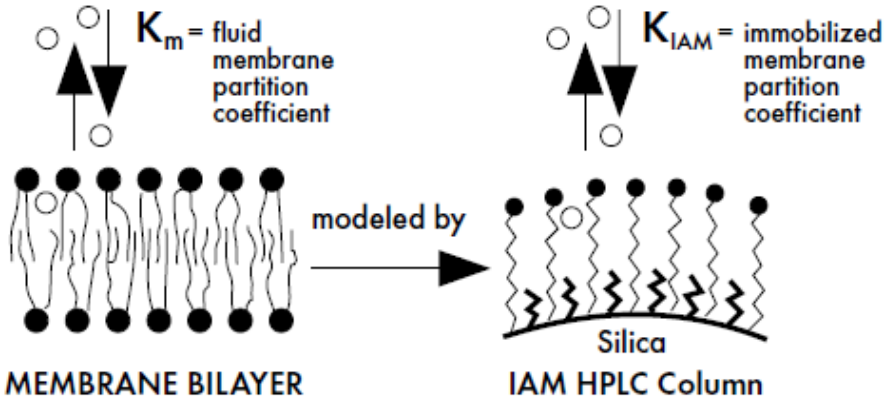


Figure 26 Fluid membrane and IAM Partitioning Measurements

To measure lipophilicity with HPLC method, the capacity factor log k 'for solutes is determinate. This is a chromatographic parameter directly related to the retention time of solute in the stationary phase used. The retention time is longer when the solute has higher affinity to the stationary phase due to its chemical nature. A non retained substance passes through the column at a time t_0, called the Void Time. The Capacity Factor describes the thermodynamic basis of the separation and its definition is the ratio of the amounts of the solute at the stationary and mobile phases. The addition of an organic modifier (methanol, ethanol or acetonitrile) to the mobile phase is needed for the elution of highly lipophilic compounds. The extrapolation has to be made to the capacity factor for a 100% aqueous phase (log K'w) to determinate the partition coefficient .

8.2.2.1 Experiemental procedure

Dilutions of 0.9 mg / ml in methanol of pinoresinol were prepared to determine the retention time on the surface of a chromatographic column IAM.PC, artificial membranes composed of phosphatidylcholine immobilized on the surface of silica. Were injected 10 µl of solution and a methanol control in a column of IAM.PC.DD2 (100 x 4.6 mm, 12 µm and 300 Å, Regis Technology, Morton Grove, IL, USA), and retention measurements were performed by HPLC using an Agilent LC 1100 series (Agilent Technologies, Inc., Palo Alto, CA, USA) equipped with a pump, autosampler, column oven and UV detector set to 280 nm. The column was eluted with a flow rate of 1.0 ml / min and the column temperature was set at 30° C.

Binary mixtures of 0.01 M phosphate buffer and acetonitrile were prepared with a volume ratios between 20 to 80% of organic modifier in phosphate buffer and adjusted to pH 7.4. Solutions were filtered and degassed in an ultrasonic water bath prior to use.

Capacity factors k'_{IAM}) of Pinoresinol, for each composition of mobile phase, was calculed using the equation 1 [223, 229]:

$$\mathbf{K'_{IAM}} = \frac{T_R - T_0}{T_0} \qquad \mathbf{(1)}$$

where T_R is the retention time of the compound tested and T_0 the dead of the column which is equal to the retention time of a substance which is not retained. For this purpose citric acid was used at 0.5 mg / ml dilution in the corresponding mobile phase composition.

Log K'_{IAM} values determinate for the respective compositions of eluent were plotted against mole fractions . Log K'_{IAMw} values were extrapoled from K'_{IAM} versus mole fractions plots to 100 % aqueous phase using the following equation 2 :

$$\mathbf{\log k'_{IAM}} = S \cdot \phi + \log k'_{IAMw} \qquad \mathbf{(2)}$$

where S is the slope of the linear regression obtained and φ the acetonitrile concentration present in the mobile phase. The partition coefficients, K_{IAM}, were calculated with the capacity factor K'_{IAMw}, the mobile phase volume Vm and the stationary phase volume Vs by following relation [223, 229]:

$$\mathbf{K_{IAM}} = \frac{Vm}{Vs} \cdot k'_{IAMw} \qquad \mathbf{(3)}$$

The volume of the stationary phase was 0.125 mL for IAM.PC.DD2 column 100×4.6 mm and the volume of the mobile phase equals:

$Vm = f t_0$

where f is the flow-rate.

8.3 Differential scanning calorimetry

Differential scanning calorimetry (DSC) is a thermodynamic technique suitable for studying phase transition of membrane bilayers with and without inserted drug molecules. It has been extensively used to investigate the thermal changes caused by the incorporation of the drugs into the membrane bylayers .

Above the chain melting transition (also known as the main-transition) temperature Tm, phospholipids form the fluid Lα phase, where the hydrocarbon chains are disordered with many trans-gauche isomerizations in their C-C bonds. Positional order in the plane of the bilayer is liquid-like in this phase. The lower temperature gel (Lβ

or Lβ′) phase is characterized by flat bilayers with the hydrocarbon chains predominantly in the fully-stretched all-trans conformation (**Fig.27**). In the Lβ′ phase the chains are tilted relative to the bilayer normal, whereas in the Lβ phase there is no such tilt. The presence of a tilt depends on the relative cross-sectional areas of the head group and chains of the lipid species .

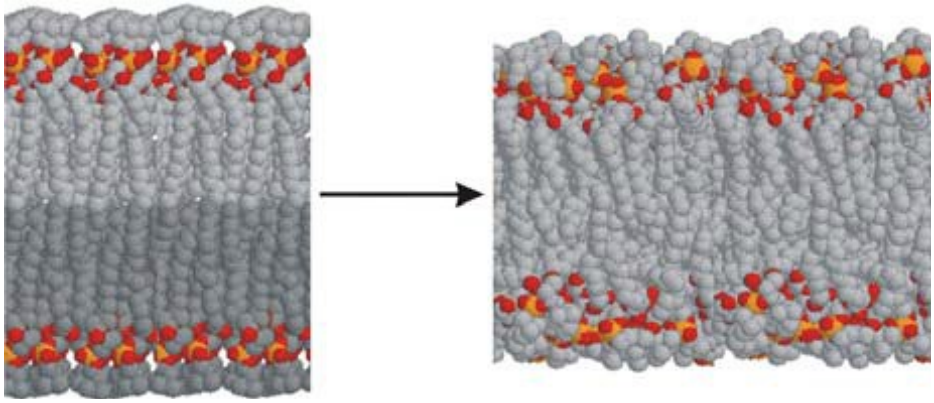


Figure 27 Gel state phase transition (left) to fluid (right) in s phosphatidylcholine bilayers caused by temperature increase

The gel to liquid-crystalline phase transition is highly "cooperative." The phase transition temperature is defined as the temperature required to induce a change in the lipid physical state from the ordered gel phase, where the hydrocarbon chains are fully extended and closely packed, to the disordered liquid crystalline phase, where the hydrocarbon chains are randomly oriented and fluid. There are several factors which directly affect the phase transition temperature including hydrocarbon length, unsaturation, charge, and headgroup species. As the hydrocarbon length is increased, Van der Waals interactions become stronger requiring more energy to disrupt the ordered packing, thus the phase transition temperature increases. Likewise, introducing a *cis* double bond into the acyl group puts a kink in the chain which requires much lower temperatures to induce an ordered packing arrangement. Some membrane phospholipids, especially phosphatidyl ethanolamines

(PEs), tend to form no-lamellar phases such as inverted hexagonal phase (H_{II}). This change in the structure of the phospholipid vesicles of the PEs is reflected by a phase transition from liquid crystalline a reversed hexagonal and occurs at higher temperatures (**Fig. 28**). This phase transition (T_H) further cooperatively also occurs although the enthalpy associated with this process is much lower than that observed in the principal phospholipid transition (Tm) .

Phospholipids such as phosphatidylcholines (PC) exhibit a modulated phase between the Lα and the Lβ′ phases at high water content. This is known as the Pβ′ or the ripple phase, and is characterized by a one-dimensional periodic height modulation of the bilayers. In this phase the hydrocarbon chains are partially ordered and have a non-zero tilt relative to the bilayer normal. The first order Lβ′ – Lα transition is well understood in terms of the melting of some of the degrees

of freedom within the hydrocarbon chains [7]. However, the first-order Lβ′ – Pβ′ transition (often called the pre transition), which occurs at a temperature slightly below

the main transition, is much less understood .

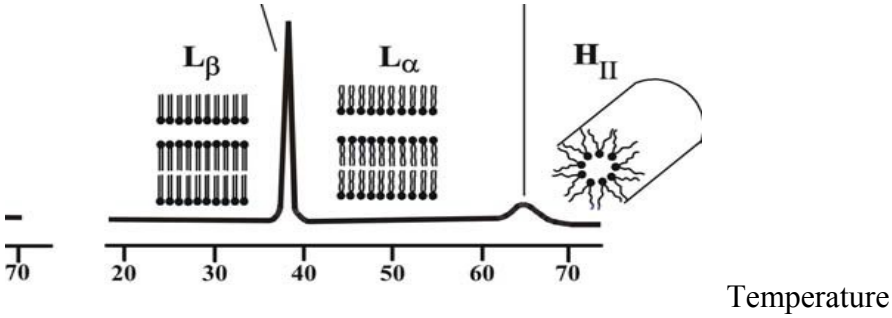


Figure 28 DEPE termogram by Differential scanning calorimetry

The DSC contains a sample cell and a reference cell that are maintained at the same temperature. As an experiment proceeds, the sample and reference cells are raised in temperature in a controlled manner such that the two cells always are maintained at the same temperature.

The power supplied to heat each cell is monitored during this process. When a phase transition occurs in the sample cell, there is a difference in the power needed to heat the two cells. The power required to maintain both cells at the same temperature is measured and converted to give an output of heat capacity versus temperature. The heat capacity versus temperature curve is analyzed to determine the transition temperature, Tm, and the calorimetric enthalpy of transition, ΔHcal (**Fig. 29**).

Figure 29 Differential scanning calorimetry

8.3.1 Experimental procedure

Chloroform/methanol solutions containing 2.4 mol of total phospholipid (DMPC, DMPG or DEPE) and the appropriate amount of pinoresinol were dried under a stream of oxygenfree

N_2 in order to obtain a thin film at the bottom of small thick-walled glass tubes. Last traces of solvent were removed by keeping the samples under high vacuum for >3 h. MLVs were formed by incubating the dried lipid on 1.6 ml of buffer (10m MHEPES, 0.1mM EDTA) with the desired pH and NaCl concentration for about 15 min at a temperature above the gel to liquid-crystalline transition (but below the temperature of the lamellar liquid-crystalline to hexagonal-H_{II} transition for DEPE containing vesicles) with occasional and vigorous vortexing. Thermograms were recorded on a high-resolution Microcal MC-2 differential scanning microcalorimeter, equipped with a DA-2 digital interface and data acquisition utility for automatic data collection. Differences in the heat capacity between the sample and the reference cell, which contained only buffer, were obtained by raising the temperature at a constant rate of 1◦C/min over the range from 8 ◦C to 45 ◦C for samples containing DMPC or DMPG, or from 15 C to 75³C for samples containing DEPE. A series of three consecutive scans of the same sample were performed to ensure scan-to-scan reproducibility. The second scan was used for transition calculations unless otherwise stated. After the thermal measurements, the phospholipid content of the sample volume was determined. The Microcal Origin software (Microcal LLC) was used for data

acquisition and analysis. The excess heat capacity functions were obtained after baseline subtraction.

8.4 Steady-state fluorescence anisotropy

When a fluorescent molecule is excited with polarized light, the resulting fluorescence is also polarized. Since the main cause of fluorescence depolarization is rotational diffusion of the fluorophore during the excited lifetime, fluorescence polarization measurements can be used to determine the rotational mobility of the fluorophore. Fluorescence anisotropy (r) is an experimental measure of the fluorescence depolarization. The lower the anisotropy value, the faster the rotational diffusion. Anisotropy measurements are made by exciting the fluorophore with polarized light and measuring the fluorescence intensity both parallel and perpendicular to the excitation polarization as shown in figure 30.

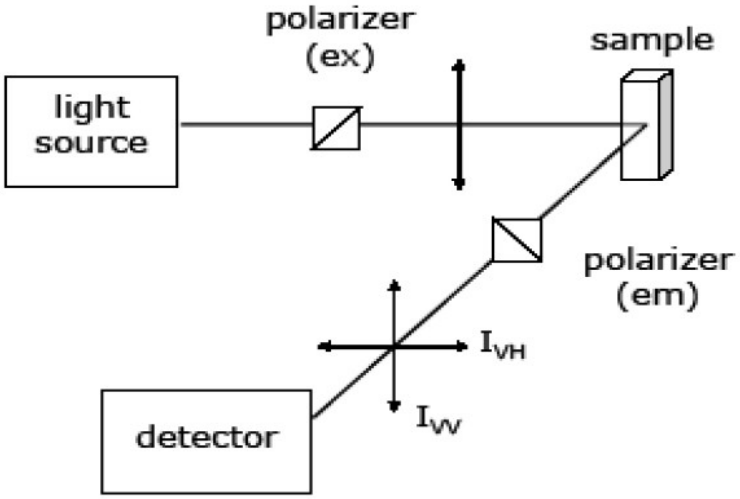


Figure 30 Schematic of a fluorometer used to make polarization/anisotropy measurements.

Specifically, the sample is excited with vertically polarized light and the vertical and horizontal emission components (IVV and IVH) are measured. Anisotropy (r) is defined by the following equation (4):

$$r = \frac{I_{vv} - GI_{VH}}{I_{vv} + 2GI_{VH}} \qquad (4)$$

where the G-factor (G) is the intensity ratio of the vertical to horizontal components of the emission when the sample is excited with horizontally polarized light (G = IHV/IHH). G is dependent on monochromator wavelength and slit widths; as long as the experimental

conditions remain unchanged, the G-factor only needs to be measured once for the experiment.

The measured anisotropy represents a weighted average of the anisotropies of the fluorophore if it is in multiple forms or environments.

Steady- state fluorescence anisotropy of DPH probe is largely determined by the structural order of the lipid bilayer, so that the microviscosity values based on these measures reflect more the alignment and packing of membrane components that mobility. Therefore, the Steady- state fluorescence anisotropy measurements give information about the structural order of phospholipid environment surrounding the probe .

To determine the degree of alteration on the packing of the membrane caused by the presence of antioxidant compound Pinoresinol we studied the variation of the steady- state anisotropy of a fluorescent lipophilic probe sensible to changes in phospholipids state.

We used 1,6-Diphenyl-1,3,5-Hexatriene (DPH), that shows a cylindrical shape , high hydrophobicity and size and for these reasons can align parallel to the phospholipids acyl chains and packed between them. According London et al. , DPH be located to 7.8 Å from the center of the bilayer.

8.4.1 Experimental procedure

DMPG, DMPC and DEPE model membranes (MLVs) containing different Pinoresinol concentrations were prepared as described for DSC measurements with 10 mM HEPES, 0.1mM EDTA and 100 mM NaCl at different pH values.

The sample pH was adjusted by addition of HCl below pH 7.4 and was controlled before and after the measurements. The phospholipid suspensions were adjusted to a final phospholipid concentration of 1mM in order to eliminate the effect of lipid dilution in the thermal behavior of

DMPG. Aliquots of DPH in *N*,*N*_-dimethylformamide (3.1×10^{-4} M) were directly added into the lipid dispersion to obtain a probe/lipid molar ratio of 1/200.

Samples were incubated for 1 h well above the gel to liquid-crystalline phase transition temperature and 2 min more at 50 ◦C for DMPG samples, and measurements were taken immediately thereafter. The steady state fluorescence anisotropy, (r), of DPH was measured in an SLM-8000C spectrofluorimeter (SLM Instruments Inc., Urbana, IL, USA). The temperature was controlled with a Varian temperature controller and the measures were done at a constant rate of 0.25 ◦C/min over the range from 10 to 55 ◦C for DMPC and DMPG and from 10 to 70 ◦C for DEPE.

The vertically and horizontally polarized emission intensities, elicited by vertically polarized

excitation, were corrected for background scattering by subtracting the corresponding polarized intensities of a phospholipid preparation lacking probes. The G-factor, accounting for differential polarization sensitivity, was determined by measuring the polarized components of fluorescence of the probe with horizontally polarized excitation ($G = I_{HV}/I_{HH}$).

Results

1. Polyphenols'quantification by Folin-Ciocalteu technique

Total phenol content of 14 different EVOOs extracts was determined using Folin-Ciocalteu technique. These olive oils derived from five different monovarietal EVOOs obtained from different geographical zones in Spain: two Hojiblanca olive oils produced in Malaga (EVOO 1) and Sevilla (EVOO 9), seven Picual olive oils produced in Malaga (EVOO 2), three from Jaen (EVOOs 4, 10 and 11), two from Granada (EVOOs 5, and 6),

and one Cordoba (7), one Cornezuelo (EVOO 3), one Manzanilla (EVOO 8), and three Arbequina olive oils (EVOOs 12, 13, and 14).

A calibration curve of gallic acid was prepared (**Fig 31**) and, then, the total phenolic content was determined by a comparison of the values obtained with the calibration curve of gallic acid. The results were expressed as gallic acid equivalents (GAE) in milligrams per L of sample.

The 14 varieties had significantly different phenolic compositions (**Table 2**), ranging from the lowest value shown for EVOO 12, i.e. 2467.54 ± 59.69, until the highest value foun in EVOO 8, i.e. 9443.42 ± 63.44.

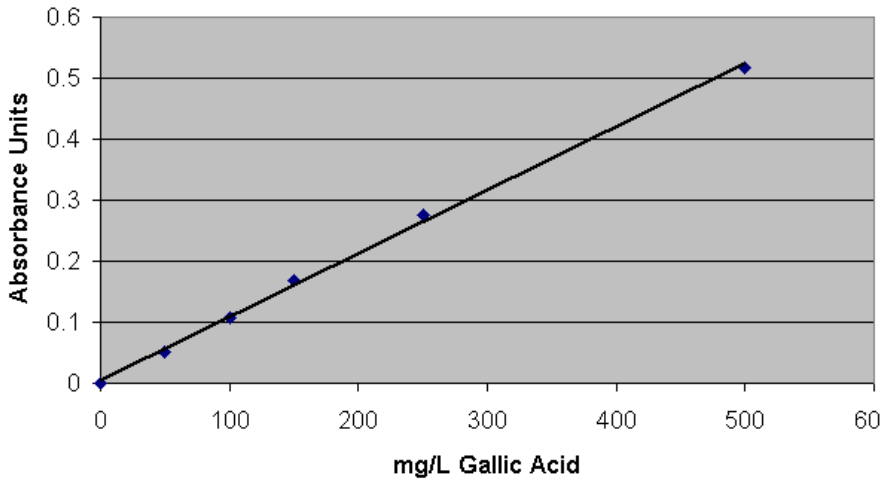


Figure 31 Acid gallic standard curve

Extract	Concentration mg/L
1	**5950 ± 433.06**
2	**7261.40 ± 175.58**
3	**6636.40 ±412.23**
4	**6314.03 ± 539.72**
5	**6259.21 ± 69.31**
6	**7327.19 ± 51.10**
7	**9064.03 ± 53.17**
8	**9443.42 ± 63.44**
9	**8767.42 ± 50.24**
10	**7572. ± 42.80**
11	**8564.03 ± 138.67**
12	**2467.54 ± 59.69**
13	**4267.98 ± 41.78**
14	**4228.50 ± 42.80**

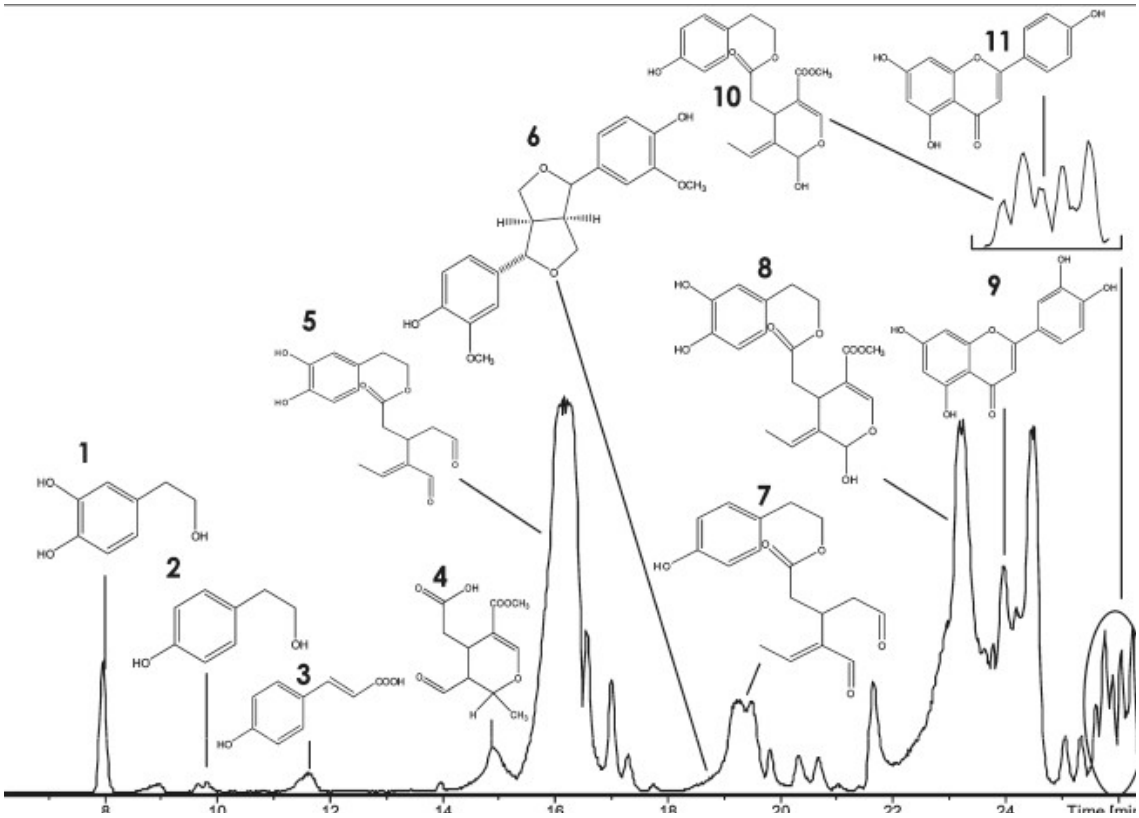
Table 2 Concentration of phenolic compounds in EVOO-PE extracts

2. Identification and quantification of the bioactive compounds in the extracts by HPLC-MS analysis

To identify and quantify the polyphenolic compounds, the analysis of the different EVOOs varieties was carried out by HPLC-ESI-TOF/MS.

As an example of the identification, **Figure 32** shows the chromatogram of EVOO 7 obtained by this method. In first instance, external standards of phenolic with known concentrations were injected into HPLC in order to set calibration curves; then samples were analyzed and their quantification was performed. The phenolic and other polar

compound concentrations were determined using the area of each individual compound (three replicates) and by interpolation in the corresponding calibration curve. Phenolic compounds such as hydroxytyrosol, tyrosol, oleuropein, luteolin, apigenin, and (þ)-pinoresinol and other more polar compounds such as quinic acid were quantified by the calibration curves obtained from their respective commercial standards. The other phenolic compounds, which had no commercial standards, were tentatively quantified on the basis of other compounds having similar structures. The detection was carried out using UV detector at two typical wavelengths characteristic of the phenolic compounds of interest (280 and 240 nm) and mass spectrometry time-of-flight (TOF). Finally, a total of 20 compounds from different families (simple phenols, flavonoids, lignans, and secoiridoids)



were identified.

Previous studies reported the strong ability to alter breast cancer cell viability by secoiridoids-rich EVOO and lignans-rich EVOO varieties. For this reason, we chose a secoiridoids-rich EVOO (EVOO7 or EVOO A) and a lignans-rich EVOO (EVOO 12 or EVOO B) for further citotoxicity studies in breast cancer cellular model.

Table 3 shows the content (in µg/mL) of each family of phenolics included in the 100% full strength stocks of 14 EVOO extracts.

Figure 32 Base peak chromatogram (BPC) of the EVOO 7 obtained by HPLC-ESI-TOF/MS. The main identified phenolic compounds are: 1, HYTY; 2, TY; 3, Vanillin; 4, EA; 5,DOA; 6, PIN; 7, D-LIG Agl; 8, Ol Agl; 9, Lut; 10, LIG Agl; 11 Apig.

	µg total phenolic contents / mL	µg phenolic alcohols/ mL	µg secoiridoids/ mL	µg lignans/ mL	µg flavonas/ mL
1	**1100.22±3.55**	**32.53±0.89**	**1043 ±4.60**	**13.51±0.04**	**11.10 ±0.23**
2	**1369.23±11.51**	**35.48±0.25**	**1321.28±11.51**	**7.064±0.06**	**5.40±0.01**
3	**1282.64±9.98**	**18.00±0.38**	**1252.89±10.13**	**4.669±0.10**	**7.074±0.01**
4	**1550.93 ±4.80**	**54.29±0.99**	**1481.79±79**	**7.87±0.0368**	**6.96±0.03**
5	**1767.56±4.63**	**48.22±0.07**	**1702.03±4.63**	**7.35±0.051**	**9.95±0.08**
6	**1663.81±1.38**	**46.165±0.15**	**1600.04±1.30**	**6.81±0.01**	**10.78±0.08**
7	**2050.73±6.07**	**43.40 ± 0.85**	**1988.01 ± 6.81**	**4.75±0.18**	**14.55 ±0.11**
8	**1622.42±25.41**	**48.21±0.54**	**1536.78±24.78**	**14.18±0.07**	**23.232±0.27**
9	**1410.66±1.39**	**33.70±0.19**	**1331.24±1.50**	**14.37±0.08**	**31.34±0.04**
10	**1484.79±17.70**	**29.84±0.07**	**1433.31±17.79**	**7.934±0.06**	**13.70±0.14**
11	**1423.17±6.22**	**47.54±0.12**	**1353.63±63**	**7.60±0.00**	**14.39±14.39**
12	**534.09 ± 2.01**	**19.21 ± 0.14**	**427.71 ± 1.59**	**69.88 ± 0.60**	**17.28 ± 0.19**
13	**983.60±31.94**	**25.33±0.11**	**890.91±29.57**	**50.62±2.28**	**16.73±0.08**
14	**1166.32±5.87**	**32.06±0.40**	**1056.28±5.30**	**57.05±0.04**	**20.91±0.24**

Table 3 Concentration of phenolic compounds in crude EVOOs

3. Cytotoxic effects of free EVOO A and free EVOO B on the viability of JIMT1 and MCF7 breast cancer cells

In a first instance, we studied the cytotoxic effects of EVOO A (EVOO 7) and EVOO B (EVOO 12) (solubilized in ethanol) in breast cancer cellular models when applied either in a free form, incorporated into liposomes and finally encapsulated into liposomes coupled to trastuzumab (immunoliposomes). JIMT1 and MCF7 breast cancer cells at a confluence of 80% were treated with different concentrations (ranging from 1-18 µg/ mL, i.e. 0.01% - 0.2% (v/v)) of EVOO A and EVOO B in order to test their efficacy. The highest solvent concentration in culture media (0.2% v/v ethanol) had no significant effects on the metabolic status of cells. Higher concentrations of EVOO extracts could not be used because ethanol resulted to be toxic when used at concentration higher than 0.2%.

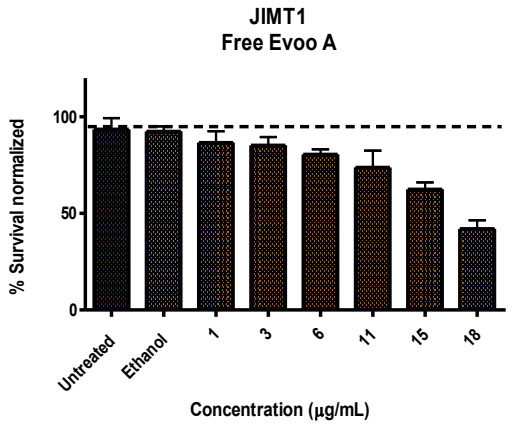
The treatments were performed for 72h, to assess a chronic effect and both cytotoxic and cytostatic effects. Exposure to EVOO A and EVOO B inhibited cell growth in the two cell lines tested in a concentration dependent manner (**Fig. 33**).

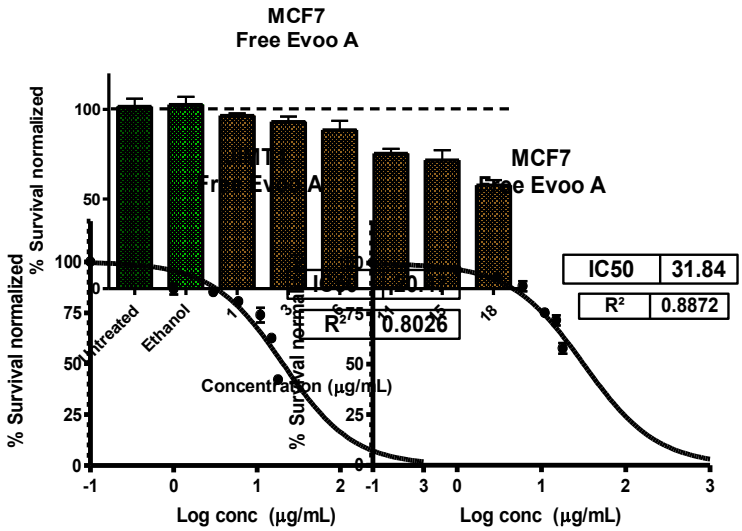
JIMT1 cells were more sensitive than MCF7 cells to both extracts and, in addition, EVOO A induced a more significant cytotoxic effect than EVOO B. Proliferation survival was

assessed by the MTT assay. The highest solvent concentration in culture media (0.2% v/v ethanol) had no significant effects on the apparent metabolic status of cells and showed no citotoxicity.

The IC50 values were determined from the survival plots, which indicate the concentration causing the death of 50% of the cell population; the coefficient of determination (R^2) ranged from 0.7 to 0.8 in all cases, indicating a highly significant correlation between experimental and fitted data points.

A





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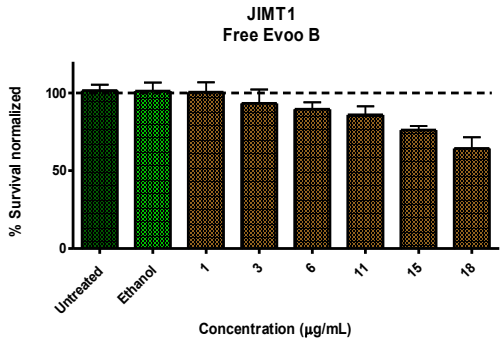
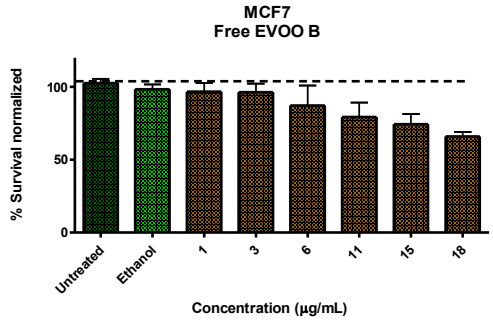
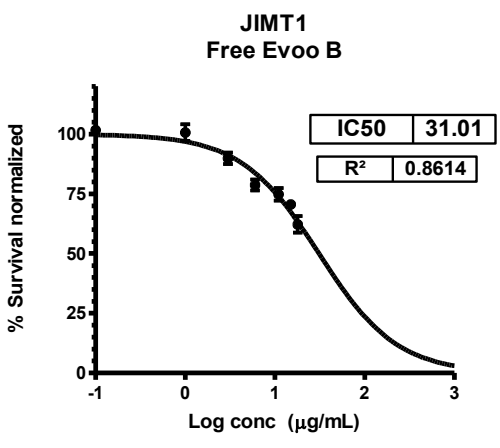






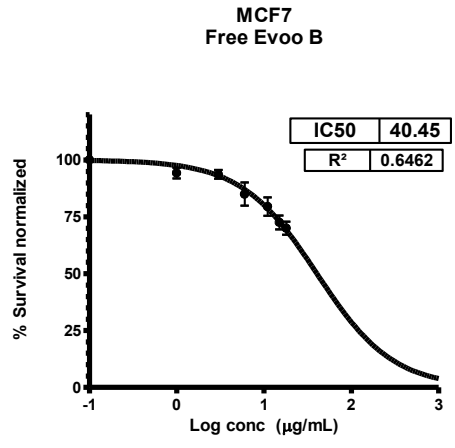


Figure 33 Effect of free EVOO A **(A)**, free EVOO B **(B)** on JIMT1 (left) and MCF7 (right) breast cancer cells proliferation after 72 hours of exposure. Percentage of cell survival after 72h of treatment with different concentrations of extracts; bars denominated untreated and ethanol correspond to cells treated only with culture medium and cells treated with the highest ethanol concentration (0.2%) used to dissolve drugs, respectively. Shown data represents the average of 3 data sets in which each condition was tested using 8 replicates**.** The error bars are the corresponding standard deviations (± SD).

Compounds	**IC_{50} JIMT1**	**IC_{50} MCF7**
Free EVOO A µg/mL	**20.47 ± 0.04979**	**31.84 ± 0.02380**
Free EVOO B µg/mL	**31.01 ± 0.02743**	**40.45 ± 0.03743**

It should be noted that EVOO concentrations higher than18 µg/ml could not be tested due to ethanol cytotoxicity. Therefore, IC50 values were estimated by extrapolation of the survival plots although full cytotoxicity was not achieved.

The estimated IC50 values ranged between approximately 20 and 30 µg/mL for free EVOO A, 30 and 40 µg/mL for free EVOO B as resumed in **Table 4.**

Results showed a greater sensitivity of both cell lines to EVOO A with a stronger cytotoxic effect in JIMT1 cells than that shown for MCF7 cells for both polyphenolic extracts.

Table 4 IC50 values of free EVOO A and free EVOO B on JIMT1 and MCF7 cell lines.

4. Optimization of drugs' encapsulation into liposomes

In order to optimize the encapsulation of EVOO polyphenols in liposomes a series of tests were made.

For the first stage, two different lipid compositions were prepared, one containing phosphatidylcholine 3 mM (EYPC) alone and another containing EYPC together with cholesterol 3 mM (80:20) in order to assess the best encapsulation system. EVOO polyphenols, 1.5% (V/V), were added together with the lipids and then mixture was hydrated. Liposomes were incubated for 1 h at 37 °C before performing the test.

After the separation of non-encapsulated extract from the encapsulated one by ultrafiltration, polyphenols were quantified in both fractions using Folin-Ciocalteu technique. Results are shown in figure 34, indicating the percentage of the polyphenols that were incorporated into liposomes (41% EYPC liposome *vs* 27% EYPC + Cholesterol

liposome). The results show that the presence of cholesterol decrease the amount of polyphenols present into the liposomes, then EYPC liposomes were selected as the system of choice for further experiments. (**Fig. 34**).

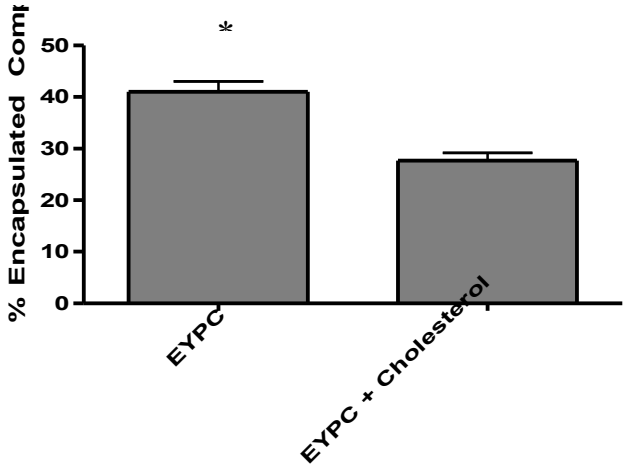


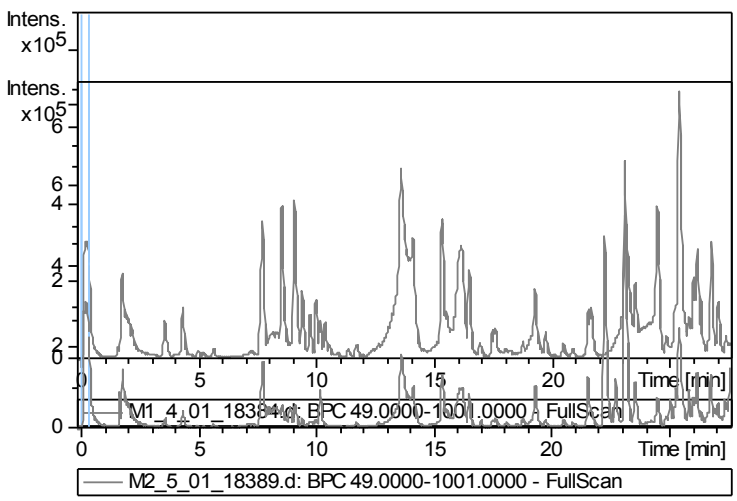
Figure 34 Percentage of polyphenols incorporated into EYPC and EYPC/Cholesterol liposomes after 1h of incubation at 37 ºC (results from Folin technique).

In order to confirm the results obtained by the Folin-Ciocalteu experiments and to identify the individual phenolic compounds of EVOO having higher affinity for the membranes, liposomes containing 1.5% (v/v) of EVOO A were prepared and separated after incubation by membrane filtration. After separation, samples were analyzed by HPLC coupled to electrospray ionization time-of-flight mass spectrometry at the University of Granada. The results are expressed as a ratio, i.e. R>1, means that this compound is found predominantly in the encapsulated form, whereas if the R<1, the non-encapsulated fraction contains the majority of this compound. R = 1 means that there is no differential distribution .

Figure 35 A, B and C and Figure 36, show the results obtained using EVOO 7 encapsulated in EYPC liposomes and, the principal compounds identified, respectively.

Table 6 summarizes the main compounds identified in the EVOO 7, including the information generated by TOF analyzer: retention time, experimental,calculed *m/z* and molecular formula.

A



B

C

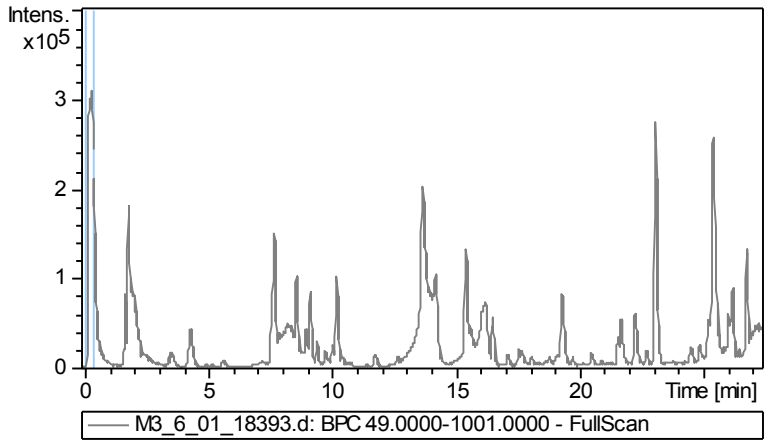
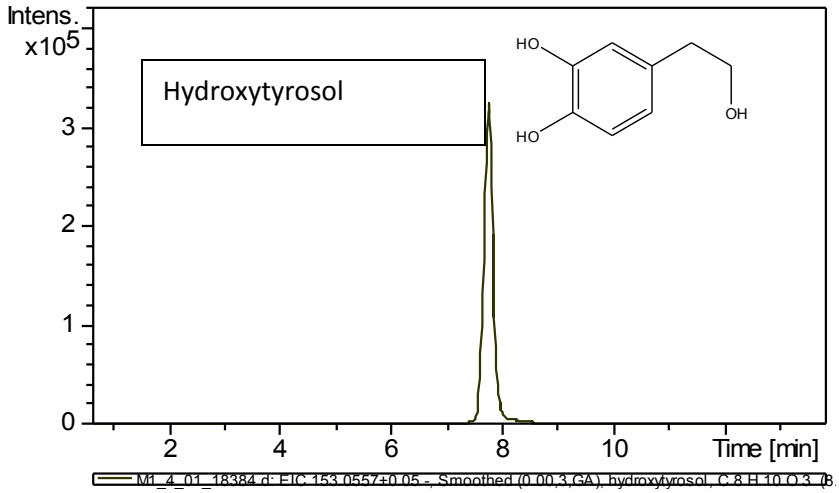
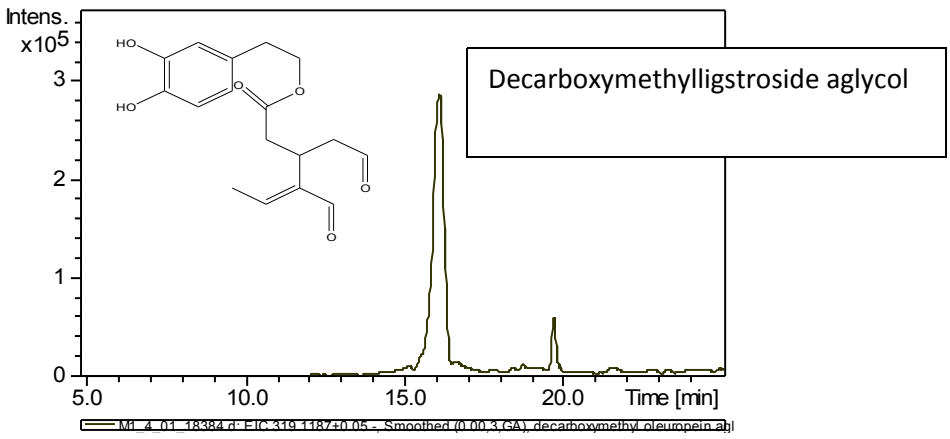


Figure 35 Base peak chromatograms obtained using EYPC liposome. A Total extract, B encapsulated extract, C no-encapsulated extract.

A



B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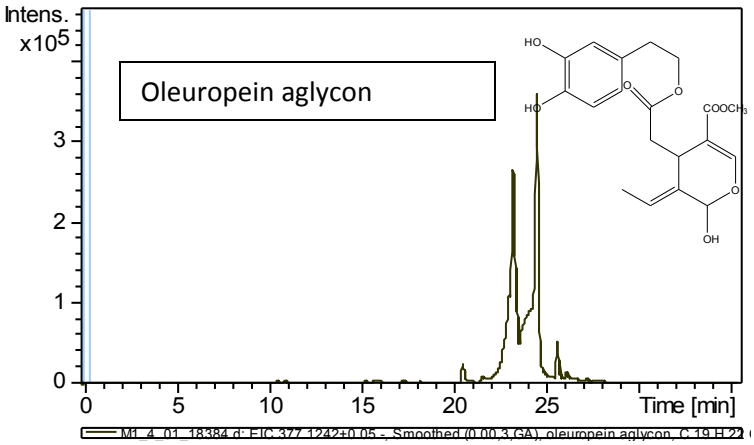
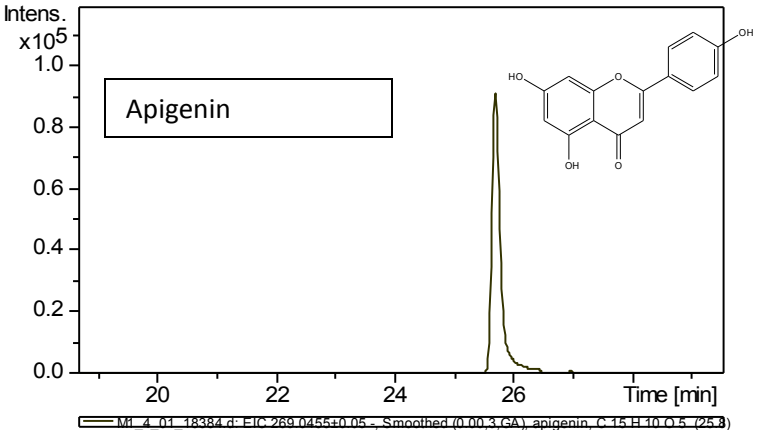
C



D



E

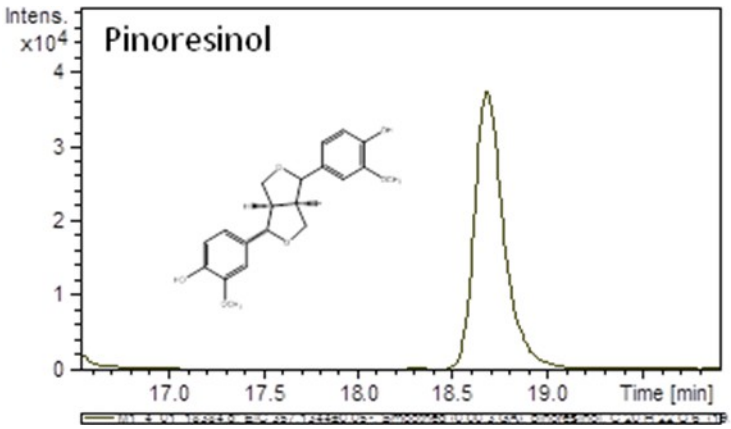


Figure 36 Principal compounds identified in EVOO 7 by RRLC-ESI-TOF. A Hydroxytyrosol, B Decarboxymethil ligstroside, C Oleuroppein aglycon, D Apigenin, E Pinoresinol,.

compd	retention time (min)	*m/z* exptl	*m/z* calcd	molecular formula
hydroxytyrosol	8	153.0556	153.0557	$C_8H_{10}O_3$
tyrosol	9.9	137.0617	137.0608	$C_8H_{10}O_2$
vanillin	11.7	151.0401	151.0401	$C_8H_8O_3$
p-coumaric acid	13.5	163.0398	163.0401	$C_9H_8O_3$
hydroxytyrosol acetate	14	195.0661	195.0663	$C_{10}H_{12}O_4$
elenolic acid	15	241.0714	241.0718	$C_{11}H_{14}O_6$
hydroxy elenolic acid	15.4	257.0667	257.0667	$C_{11}H_{14}O_7$
decarboxymethyl oleuropein aglycon	16.3	319.1193	319.1187	$C_{17}H_{20}O_6$
hydroxy D-oleuropein aglycon	16.6	335.1142	335.1136	$C_{17}H_{20}O_7$
syringaresinol	18.2	417.1562	417.1555	$C_{22}H_{26}O_8$
pinoresinol	18.8	357.1349	357.1344	$C_{20}H_{22}O_6$
decarboxymethyl ligstroside aglycon	19.2	303.1236	303.1229	$C_{17}H_{20}O_5$
hydroxy D-ligstroside aglycon	19.9	319.1190	319.1187	$C_{17}H_{20}O_6$
10-hydroxy oleuropein aglycon	23	393.1200	393.1191	$C_{19}H_{22}O_9$
oleuropein aglycon	23.2	377.1247	377.1242	$C_{19}H_{22}O_8$
luteolin	23.7	285.0397	285.0405	$C_{15}H_{10}O_6$
methyl D-oleuropein aglycon	25.4	333.1346	333.1344	$C_{18}H_{22}O_6$
ligstroside aglycon	25.6	361.1310	361.1293	$C_{19}H_{22}O_7$
apigenin	25.8	269.0448	269.0451	$C_{15}H_{10}O_5$
methyl oleuropein aglycon	26.2	391.1412	391.1398	$C_{20}H_{24}O_8$

Table 6 Main Phenolic Compounds Identified in a Representative Extract of Picual EVOO by RRLC-ESI-TOF

The results indicate that the majority of polyphenols are found in the liposomes fraction in a high proportion. Nevertheless, some differences were found in relation to the class of compound and its hydrophobicity.

Phenylethanol derivatives (tyrosol, hydroxytyrosol and its acetate derivative) were either equally distributed or in a higher degree in the aqueous fraction probably due to their polar character.

Among the lignans, acetoxypinoresinol was especially increased in the liposome fraction followed by pinoresinsol, whereas syringaresinol showed a lower affinity for the liposomes. Regarding secoiridoids, methyl oleuropein aglycone, oleuropein aglycone isomers I and II and 10-hidroxy oleuropein aglycone had a high affinity for the liposome fraction. In contrast, decarboxymethyl secoiridoids showed lower concentration in liposomes than the carboxylated forms. The flavone luteolin showed a very strong affinity for the liposome fraction followed by apigenin.

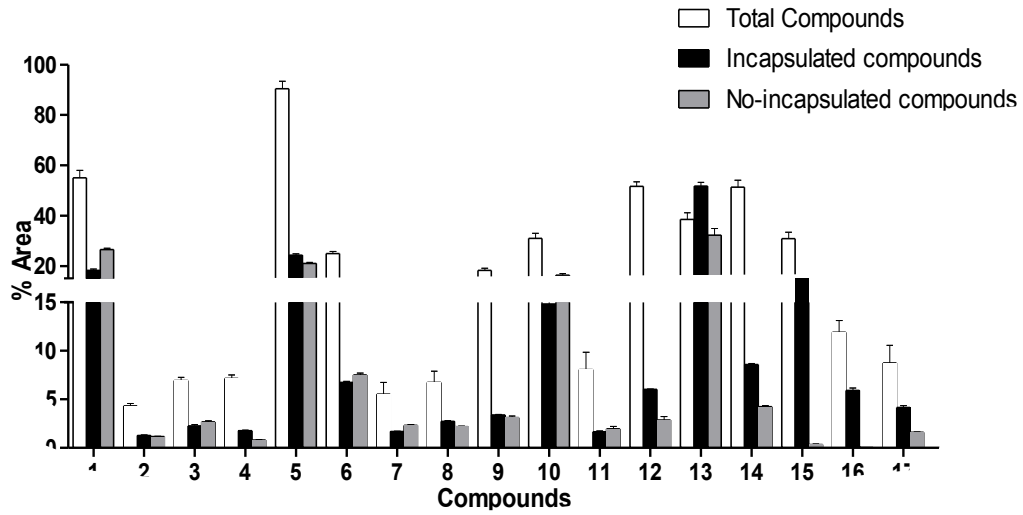
In general, most phenolic compounds were found in higher concentration in EYPC liposomes than in EYPC/Chol liposomes **(Fig 37 A and B and table 7)**, confirming the results obtained using in previous experiments using Folin-ciocalteu measurement.

In particular the highest level of encapsulation was observed for luteolin (ratio EYPC 73.5 vs 59 EYPC/Cholesterol), apigenin (ratio EYPC 5.3 vs 5.8 EYPC/Cholesterol), acetoxypinoresinol (ratio EYPC 4.18 vs 2.1 EYPC/Cholesterol), 10-hydroxy oleuropein aglycon **(**ratio EYPC 2.5 vs 1.5 EYPC/Cholesterol), oleuropein aglycon isomer II **(**ratio

EYPC 3.9 vs 2.02 EYPC/Cholesterol), methyl oleuropein aglycon **(**ratio EYPC 6.37 vs 2.5 EYPC/Cholesterol).

Based on these results, EYPC liposomes were chosen as optimal model systems for the subsequent experiments.

A



B

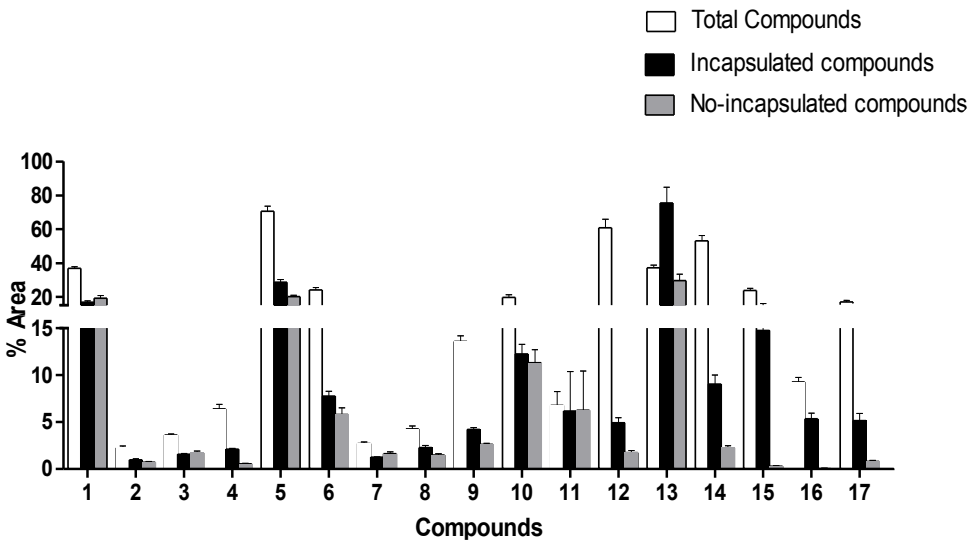


Figure 37 Percentage of polyphenols incorporated into EYPC **(A)** and EYPC/Cholesterol **(B)** liposomes

Compound	Ratio	
	EYPC	EYPC/Cholesterol
1. Hydroxytyrosol	0.89	0.6
2. Tyrosol	1.3	1
3. Hydroxytyrosol acetate	0.8	0.8
4. Hydroxy elenolic acid	4.18	2.1
5. Decarboxymethyl oleuropein aglycon	1.42	1.14
6. Hydroxy D-oleuropein aglycon isomer 2	1.3	0.8
7. Syringaresinol	0.7	0.6
8. Pinoresinol	1.5	1.2
9. Decarboxymethyl ligstroside aglycon	1.5	1.06
10. Acetoxypinoresinol	1.08	0.8
11. OH-decarboxymethyl ligstroside aglycon	0.99	0.8
12. Oleuropein aglycon isomer I	2.8	2.14
13. 10-hydroxy oleuropein aglycon	2.55	1.5
14. Oleuropein aglycon isomer II	3.99	2.02
15. Luteolin	73.5	59
16. Apigenin	5.3	5.8
17. Methyl oleuropein aglycon	6.37	2.5

Table 7 Ratio values of polyphenols incorporated in EYPC and EYPC more cholesterol at 37°C for 1 h of incubation (Results by HPLC) when incubated at 37ºC for 1h (Results by HPLC)

For the second test, the optimal incubation temperature of the mixture of liposomes and extracts in order to achieve and efficient encapsulation level was studied. EYPC liposomes 3 mM were incubated with EVOO polyphenols 1.5% (V/V) and were allowed to reach equilibrium in experiments at different temperatures, i.e : 37 °C and 42 °C. The aim was to evaluate what temperature favoured the drugs' encapsulation when the compounds were

added. After 1h of incubation the concentration of compounds incorporated into liposomes was determined by Folin-Ciocalteu technique. Results are shown in the **figure 38** indicating the percentage of the compounds incorporated into liposomes.

Although the optimal condition was found to be 37 °C, small but significant differences were detected between the two tests (43.3% EYPC liposome incubated at 37° C *vs* 37.6% EYPC liposome incubated at 42 °C).

Based on the results, 37 ºC was chosen as optimal temperature for the following experiments.

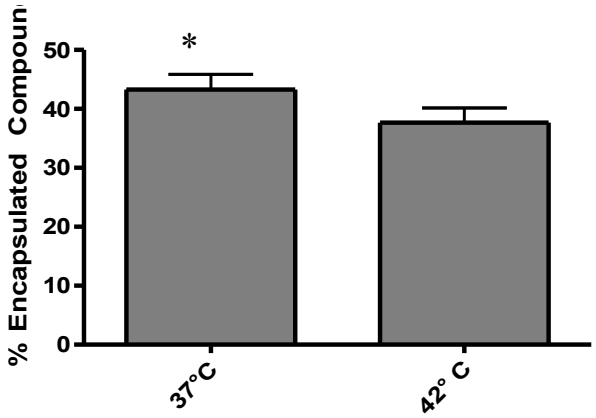


Figure 38 Percentage of polyphenols incorporated in EYPC liposome preparations at two different temperatures for 1 h of incubation.

To improve the percentage of EVOO polyphenols encapsulation, additional experiments were performed, where increasing amounts of extract were added: i.e. 1,5% and 3% (V/V), while maintaining the EYPC liposome concentration (3 mM). The best results were obtained with smaller concentration of extract (42.6% EYPC liposome more 1.5 % (V/V) of extract *vs* 30.3% EYPC liposome more 3 % (V/V) of extract.) **(Fig 39).**

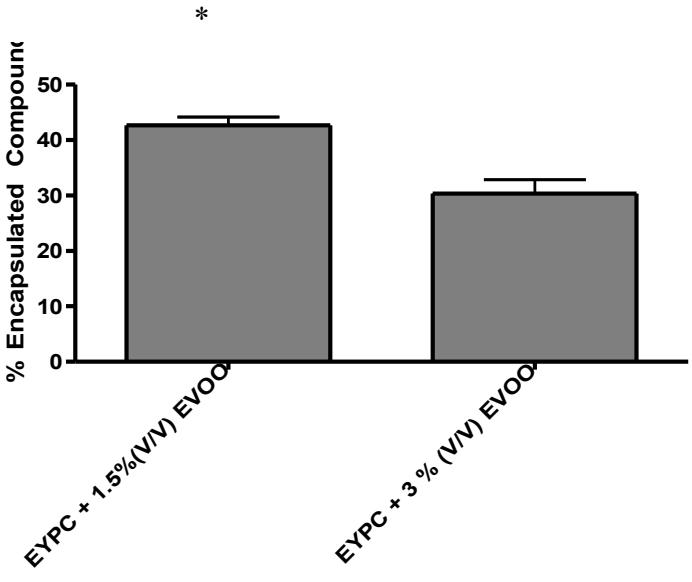


Figure 39 Percentage of polyphenols incorporated into EYPC liposomes containing different concentrations of EVOO extracts.

5 . Cytotoxic effect of EVOO A or EVOO B incorporated into liposomes

Preliminary evidences in breast cancer cell models pointed out the potential efficacy of EVOO polyphenols for breast cancer treatment. This fact along with the pharmacological safety of these compounds make them suitable for the treatment of the aforementioned condition. Regardless of the route of administration, the efficacy of these compounds depends on their bioavailability and their capability to reach their cellular targets. Indeed, only a small proportion of molecules administered orally are absorbed, because of insufficient gastric residence time, low permeability and/or low solubility. The efficacy of drugs and chemopreventive compounds when administered intravenously requires the formulation of a finished protective product able to maintain their structural integrity until their administration, increase its water solubility and bioavailability and convey it precisely towards a physiological target.

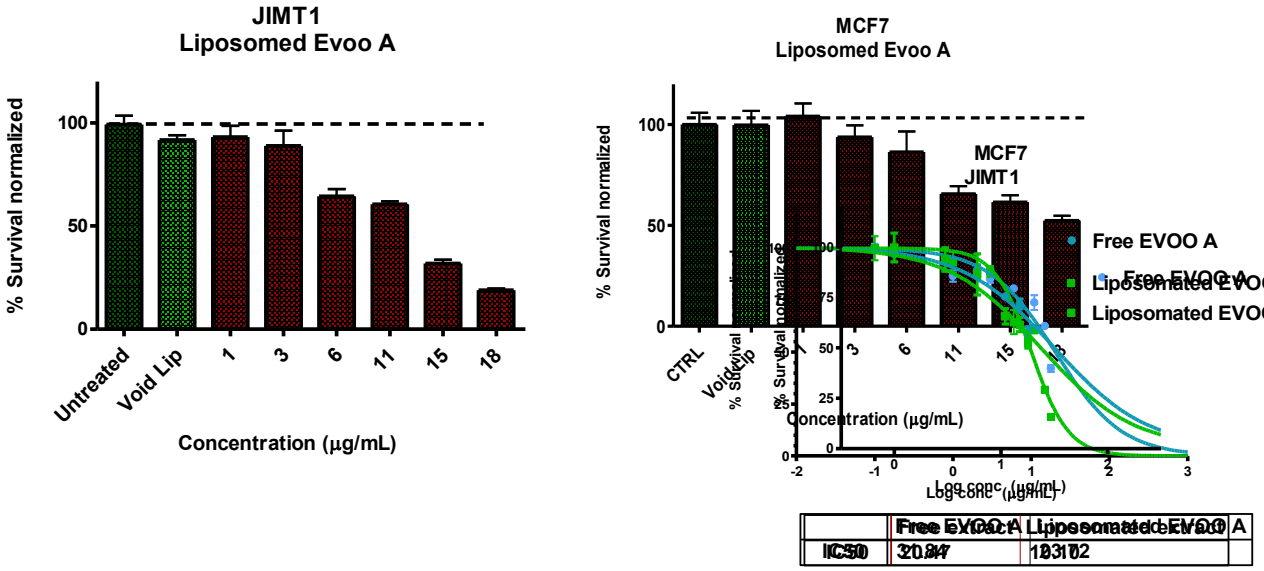
One of the possible strategies to improve their bioavailability is to encapsulate them into liposomes in order to increase blood circulation, reduce clearance and to maintain higher concentrations in their respective targets.

In a first instance, we performed the cytotoxicity assays on the two breast cancer cell models using the same bioactive compounds encapsulated into liposomes. The aim of these experiments was to evaluate a potential IC50 decrease due to their incorporation into liposomes compared to the free compounds.

As shown in **Fig. 40,** liposomal formulations showed a significant decrease of IC50 values in all treatments and consequently, an improvement of therapeutic index, i.e. achieving the same effect but using lower concentrations of drugs. Incorporation of EVOO A and EVOO B into liposomes improved its IC50 value compared to free compounds both in MCF7 and JIMT1 cells. The result was even better for EVOO A since IC50 decreased of 50% in JIMT1 and 26% in MCF7.

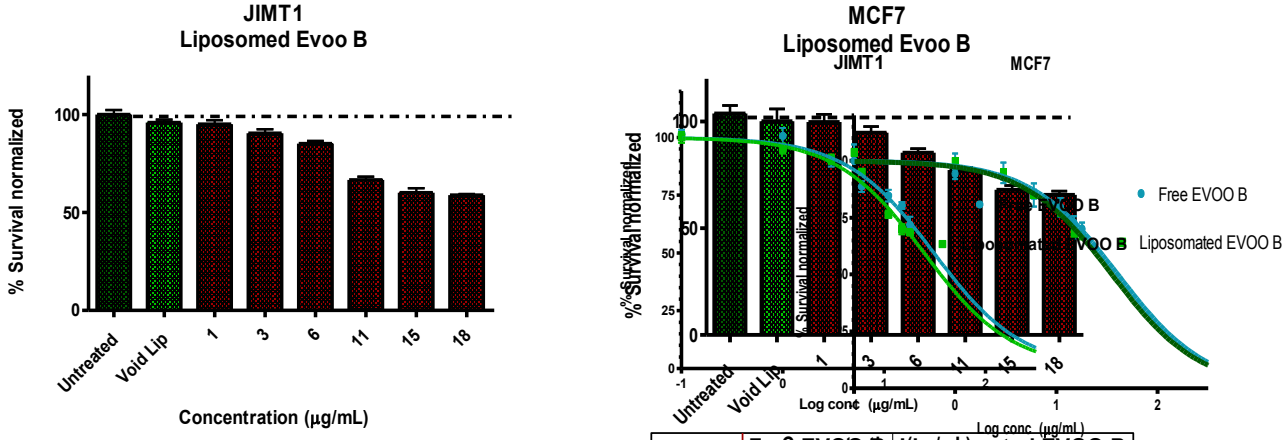
Moreover, JIMT1 cells were a little more sensitive to both EVOO A and EVOO B than MCF7 cells when these compounds were incorporated into liposomes.

A



	Free EVOO A	Liposomated EVOO A
IC50	20.47	19.02

B



	Free EVOO B	Liposomated EVOO B
IC50	31.01	24.79
IC50	40.45	35.41

Figure 40 Comparative effect of free and liposomed preparations of (A) EVOO A and (B) EVOO B and on JIMT1 and MCF7. Cell survival after 72h of treatment with different drugs' concentrations; untreated represents negative control cells treated only with DMEM; void liposomes indicates cells treated with the higher concentration of void liposomes used (0.5 mM EYPC).

6. Determination of liposome's particle size

In order to check the unilamellar character of the liposomes and the absence of aggregation, all liposomes preparations were characterized in their particle size.

Determination of size was performed using the technique of dynamic light scattering as described in Material and Methods section. Figure shows the average diameter of an typical EYPC liposome preparation lacking EVOO polyphenols and another containing 3% of EVOO A. As expected, the diameter (Z) of preparations were around 100 nm with a polydispersity (P.D.I.) value below 0.2 indicating the reliability of the measurement (**Fig 41 A and B**). Therefore, the presence of the EVOO polyphenols did not apparently affect vesicles size and morphology.

Measurements of the diameter of other types of liposomes containing cholesterol, negatively charged phospholipids or non-lamellar phospholipids were made too in the presence and in the absence of the EVOO polyphenols: EYPC/Cholesterol (80/20), EYPC/PS (80/20), EYPC/Cardiolipin/PE (75/5/20) and EYPC/PE (80/20). In all cases a small decrease of the liposome size was observed after filtration of the liposomes through 100 nm pore size filters, compared to the liposomes containin EYPC, However, no significant differences in size were observed between the liposomes containing the polyphenols and those lacking them.

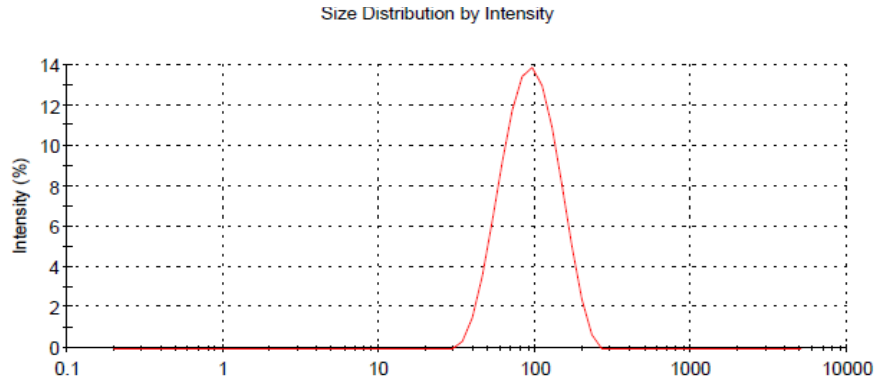


Figure 41 A Example of size distribution vs intensity, obtained from the EYPC void liposome preparation with Z= 98,87 d. nm and PDI=0.142

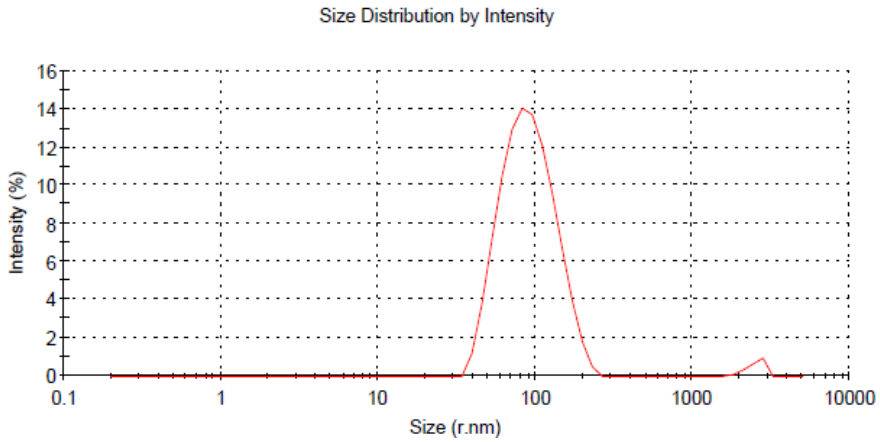


Figure 41 B Example of size distribution vs intensity, obtained from the EYPC liposome preparation containing EVOO A with Z= 98,87 d. nm and PDI=0.142

Lipidic composition	Polydispersity index		Diameter (nm)	
	Void liposome	Liposome + EVOO A	Void liposome	Liposome + EVOO A
EYPC	0.142	0.192	98.87	95.02
EYPC/Cholesterol	0.095	0.165	72.28	65.40
EYPC/PS	0.165	0.137	70	68
EYPC/PE	0.219	0.136	70.09	71.13
EYPC/Ca/PE	0.156	0.134	76.76	66.3

Table 8 Polydispersity and Diameter values of the different liposomes preparations in the presence and in the absence of EVOO polyphenols.

7. Expression of HER2 on breast cancer cell lines

7.1 Detection of HER2 by Western blot

One of the targets of the project was to compare the cytotoxicity of various phytochemicals against breast cancer cells either in their free form, incorporated into liposomes or incorporated into anti-HER2 immunoliposomes. Then, first task was to screen a variety of breast cancer cell lines in order to find two opposite models for low and high HER expression levels to test the efficacy of anti-HER2 immunoliposomes. After this, we found two cell lines with very significant differences in their HER expression levels. For this purpose western blot and flow cytometer analysis were used. We chose the trastuzumab-resistant JIMT1 cells and, as control line, MCF7 that was substantially HER2-negative. Total protein lysates from the two cell cultures were made and then, these lysates were separated by SDS-PAGE and examined by Western blot. HER2 was detectable in JIMT1 cells, whereas as expected, it was almost negligible in the lysates from MCF7 cells (**Fig. 42**). The figure 42 shows, at left, HER2 expression levels obtained by WB, while, at right, the quantification of the protein bands by densitometric analysis is shown (using the software Image J, web link: http://rsbweb.nih.gov/ij/).

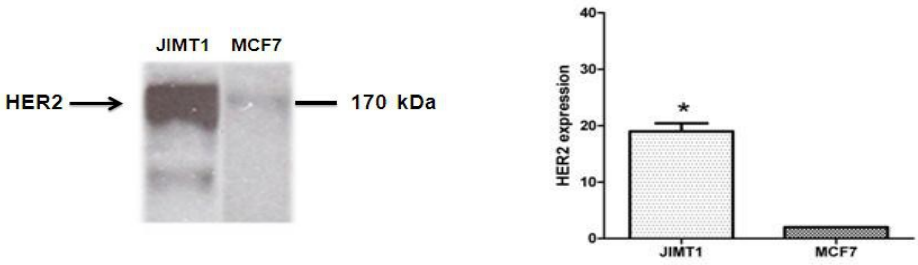


Figure 42 Western blot anddensiometric analysis of HER2 expression Whole lysates from MCF7 and JIMT1 cells were prepared and HER2 expression was quantified. Asterisk represents significant difference relativeto MCF7 cells (*p <0.05).

7.2 Detection of membrane surface HER2 by Flow Cytometry

Total amount of the protein was detected and quantified by Western blot, but to verify if the HER2 protein detected in WB assays, expressed in breast cancer cells, was present on cell surface, flow cytometry was used. In this way, we assured that immunoliposomes coupled to trastuzumab would be able to recognize cell surface HER2 and cancer cells would be properly targeted.

JIMT1 and MCF7 cells were stained with an anti-HER2/neu antibody that binds HER2 on the C-terminal portion of the extracellular domain, as previously described in materials and methods section. As shown in **Fig. 43** there was a modest binding of the antibody to MCF7 cells, whereas a more significant binding was evident in the case of JIMT1 cell line.

This was in agreement to that predicted from western blot analysis.

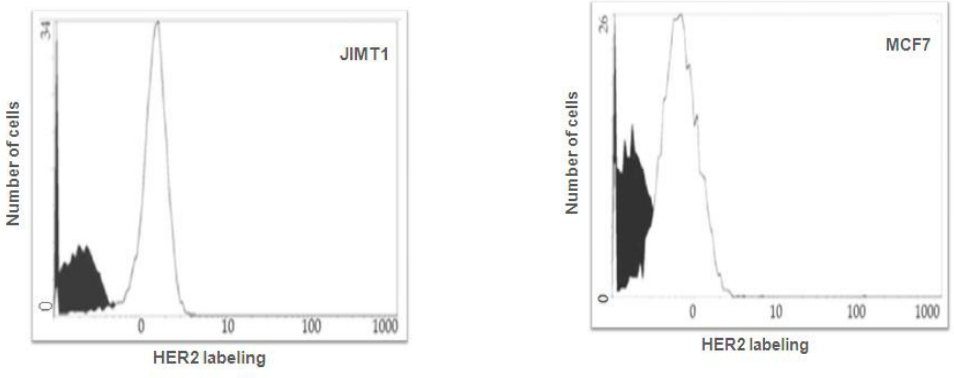


Figure 43 Flow cytometry analysis of HER2 expression Flow cytometry analysis on cells stained with an anti-HER2/neu antibody. Filled peaks of each histogram represent control cells stained only with the secondary antibody (anti-rabbit-FITC).

8. Comparative effect of the three delivery systems: free compounds, liposomes and immunoliposomes

Liposomes may represent a very efficient delivery tool. They may act as a source from which the entrapped compound or compounds are slowly released over time. This release process is used to maintain therapeutic drug levels in the bloodstream or at the local administration site for prolonged periods of time.

Despite advantages they offer, liposomes have a relevant limitation: they are not always selective for the selected targets. For this reason we used immunoliposomes directed against HER2, which is overexpressed in a high percentage of breast cancers and other cancer cell types and play an important role in the pathogenesis of breast and other cancers. The immunoliposome system was composed of different parts: (a) trastuzumab, the humanized anti-HER2 antibody; (b) sterically stabilized liposomes containing PEG; (c) the encapsulated anticancer agents. Anti-HER2 immunoliposomes were constructed by conjugation of liposomes to anti-HER2 antibody (trastuzumab) and incorporating the anticancer compounds as described in methods section. Then unbound antibody and, no-encapsulated compounds were separated by size exclusion chromatography.

Once immunoliposome systems were formed, we tested them on two different breast cancer models: JIMT1 and MCF7 cell lines. JIMT1 is a HER2-overexpressing cell line, while MCF7 cell line is substantially HER2-negative, thus the need to introduce a positive and a negative control for HER2 expression to test for selectivity of the immunoliposomes.

Cells were treated for 72 hours and also in this case, a dose-dependent response was evident (**Fig. 44**). From the graphs presented it's clear that the system based on immunoliposomes was significantly more effective than the system in which the compounds were added in their free form and slightly more effective than the system where liposomes lacking antibody were used.

Furthermore, both extracts induced a higher cytotoxic effect on JIMT1 cell lines, than on MCF7 cell lines. Using immunoliposomes loaded with EVOO A in JIMT1 and MCF7, decreases of 70% and of 49% in IC50 values were observed respectively, when these were compared with the system where free extract was used (**Fig. 44 A**).

Regarding EVOO B (**Fig 44 B)** the use of immunoliposomes induced decreases in IC50 values of 45% and 27% for JIMT 1 and MCF 7 cell lines respectively when compared with free polyphenol extract.

Therefore, in all cases the use of liposomes decreased the IC50 values compared to the free polyphenols and immunoliposomes induced even a higher decrease in these values compares to liposomes, hence increasing therapeutic index.

A

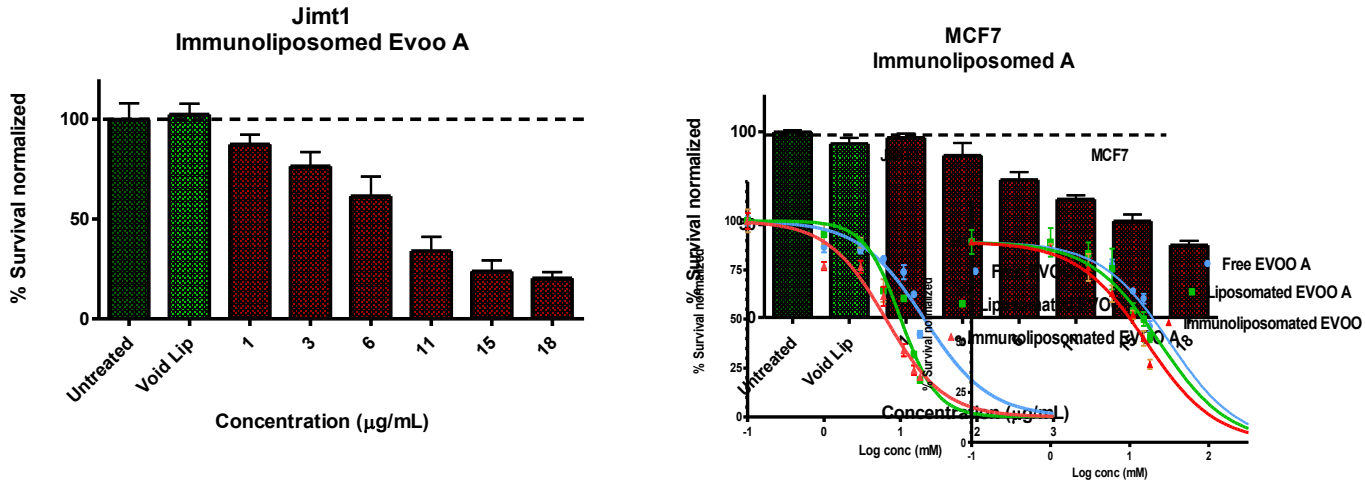
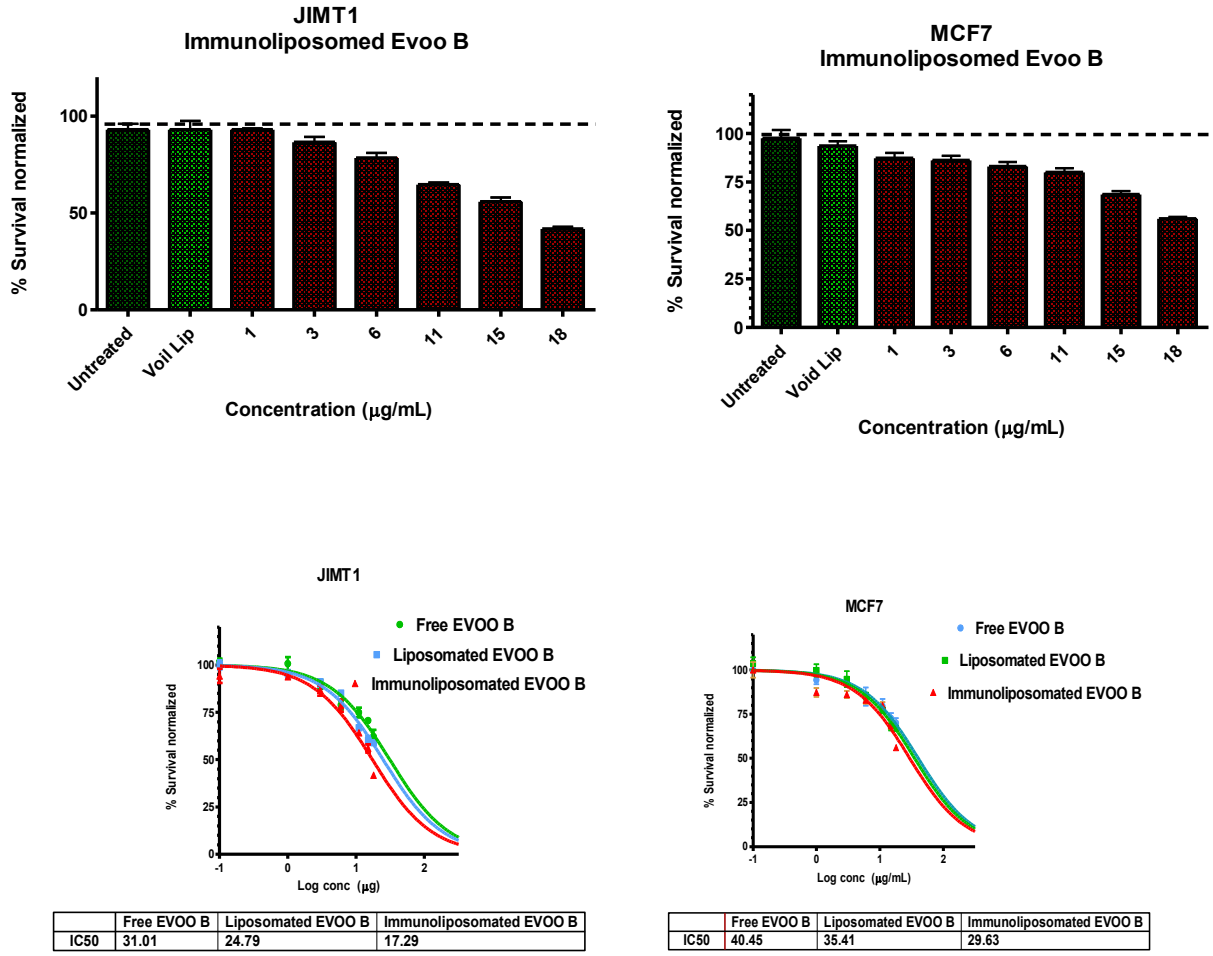


Figure 44 A. Comparative effects of the three systems: free compounds, liposomed and immunoliposomed EVOO A. Percentage of cell survival after 72h of treatment in free, liposomed and immunoliposomed systems containing EVOO A .

Compounds	**IC 50 JIM T1**	**IC 50 MCF 7**
Free EVOO A µg/mL	**20.47 ± 0.04979**	**31.84 ± 0.02380**
Liposomed EVOOA µg/mL	**10.10 ± 0.02432**	**23.72 ± 0.05384**
Immunoliposomed EVOO A µg/mL	**6.44 ± 0.03328**	**16.21 ± 0.03203**

Table 9 IC50 values of free, liposomed and immunoliposomed EVOO A on JIMT1 and MCF7 cells

B



	Free EVOO B	Liposomated EVOO B	Immunoliposomated EVOO B
IC50	31.01	24.79	17.29

	Free EVOO B	Liposomated EVOO B	Immunoliposomated EVOO B
IC50	40.45	35.41	29.63

Figure 44 B Comparative effects of the three systems: free compounds, liposomed and immunoliposomed EVOO B. Percentage of cell survival after 72h of treatment in free, liposomed and immunoliposomed systems containing EVOO B.

Compounds	**IC 50 JIM T1**	**IC 50 MCF 7**
Free EVOO B µg/mL	**31.01 ± 0.02743**	**40.45 ± 0.03743**
Liposomed EVOO B µg/mL	**24.79 ± 0.01981**	**35.41 ± 0.02885**
Immunoliposomed EVOO B µg/mL	**17.29 ± 0.02509**	**29.63 ± 0.03163**

Table 10 IC50 values of free, liposomated and immunoliposomated EVOO B on JIMT1 and MCF7 cells

There are different classes of polyphenols in the EVOO extracts used in this study: phenylethanols, secoiridoids, lignans and flavones. One plausible approach would be to

study the incorporation in membranes of one single compound and to test its cytotoxic effects on cellular models. Previous studies have postulated that secoiridoids and lignans may be the most active compounds accounting for the anticancer effects of these compounds. Among all the secoriridoids and lignans found in the extracts only oleuropein and pinoresinol are commercially available as pure standards in order to establish studies with model membranes and cellular studies. Oleuropein has been thoroughly study both in cancer cells and model membrane studies, we have selected pinoresinol to perform studies on its incorporation in model membranes.

9. Measurement of spectroscopic properties of pinoresinol and determination of its membrane partition coefficient

The spectroscopic characteristics of pinoresinol (**Fig.45**) were analyzed in water and methanol. UV spectra in methanolic solution of purified pinoresinol showed an absorption spectrum with maximum at 280 nm typical for a phenolic ring system (**Fig. 46**). In order to determinate if pinoresinol intrinsic fluorescence properties would be appropriate for studying its interaction with membranes, its fluorescence excitation and emission spectra were studied. The fluorescence excitation spectrum was identical to the UV absorption spectrum with maxima located at 280 nm in water and MeOH. Fig **47** shows the fluorescence emission spectra of pinoresinol at 280 nm of excitation. These emission spectra were similar in water and in methanol (maximum at 310 nm).

Nevertheless, the intensity of the spectra was stronger in the organic solvent, methanol, than in water. This fact may indicate that pinoresinol may present similar fluorescence properties when incorporated into phospholipid membranes.

We tried to calculate the phospholipid/water partition coefficient of pinoresinol, K_p using its fluorescence properties in lipophilic environments (DMPC and DMPG vesicles) but no change in the intrinsic fluorescence of pinoresionl was observed.

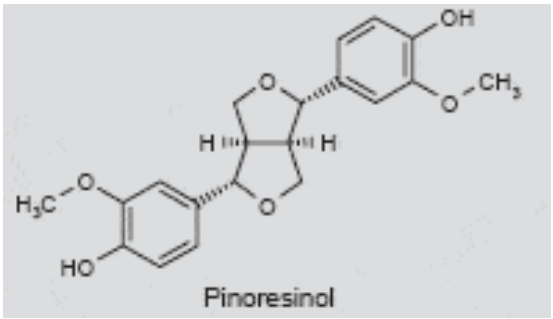


Figure 45 Structures of pinoresinol

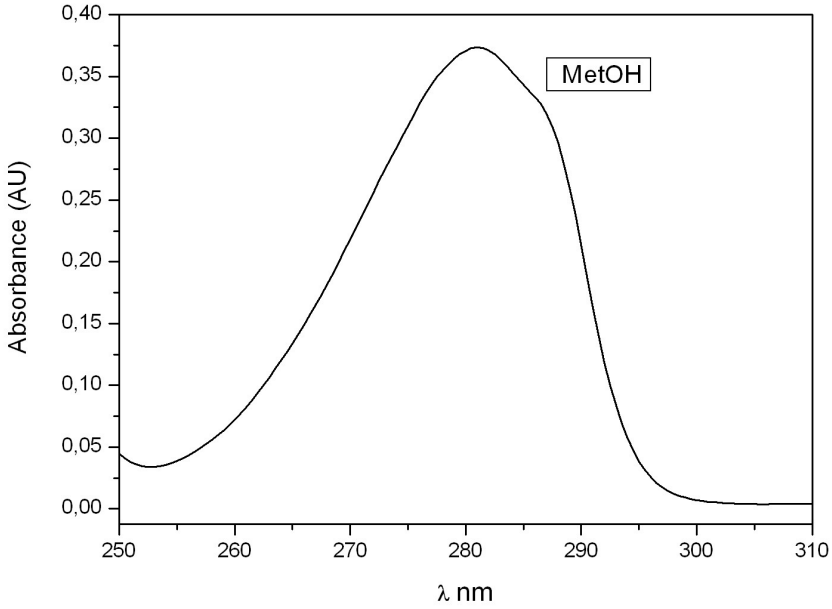


Figure 46 UV spectra of pinoresinol in methanol

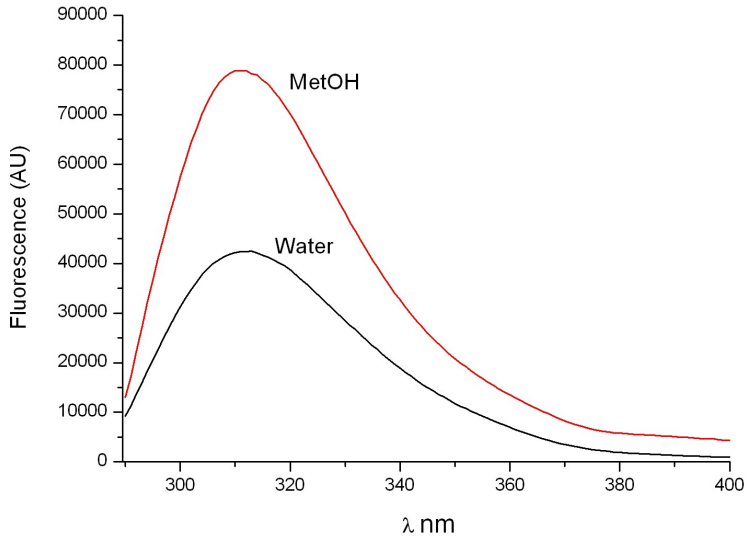


Figure 47 Fluorescence emission spectra of pinoresinol in methanol and water

10. Partition value of pinoresinol into phospholipid bilayers studied by IAM-HPLC

To describe a chemical's lipophilic or hydrophobic properties of pinoresinol, partition coefficient was determinated using an alternative technique such as immobilized artificial membrane chromatography.

The immobilized articial membrane (IAM) columns mimic the lipid environment of a fluid cell membrane on a solid matrix.

HPLC partition method permits to calculate the partition coefficient of compounds and drugs and compare it to traditional octanol/water system .

To study the retention characteristics of a compound on IAM-column, binary mixtures of phosphate buffer with an organic modifier like acetonitrile are applied.

The capacity factor K'_{IAM} was determined from the elution times of pinoresinol and for each mobile phase composition as indicated in materials and methods section.

Log K'_{IAM} values were plotted against the respective amounts of organic modifier used as mole fractions for the extrapolation of k'_{IAMw} **(Fig 48 and table 11)**.

The absence of organic solvent (100% aqueous phase) simulates conditions in which the membrane –compound partition will occur, so, the value of k'_{IAM} extrapolated by linear regression to 0% of acetonitrile allows the calculation of the partition coefficient k'_{IAM} of a given compound to lipid phases (k'_{IAMw}). In the 20-40% acetonitrile concentration, pinoresinol exhibited a retention time that decreases with increasing acetonitrile concentration in the mobile phase, which means that this compound has an intermediate hydrophobicity.

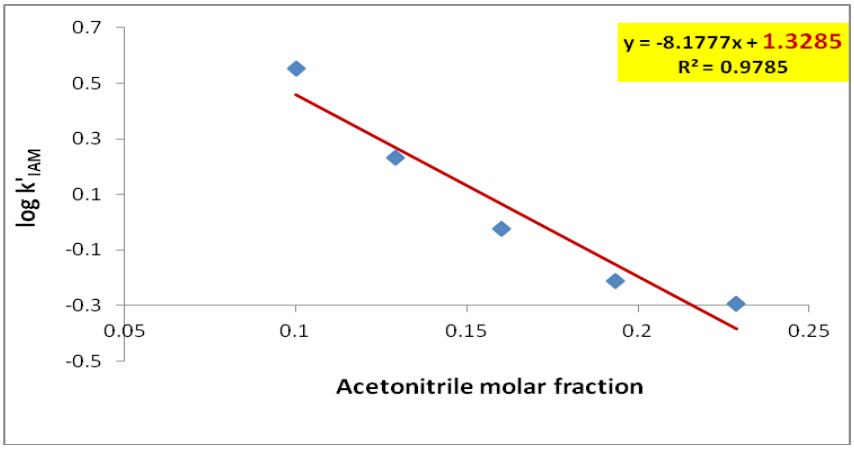


Figure 48 Linear regression equations obtained from the representation of log k'_{IAM} Vs acetonitrile%

Table 11: Linear regression equations obtained from the representation of log k'_{IAM} Vs acetonitrile %				
	Log k'_{IAMw}	***S***	**Φ range (%,v/v)**	**r^2**
Pinoresinol	**1.3285**	**-8.1777**	**20-40**	**0.9785**

The linear correlation obtained from the plot of k'_{IAM} parameter vs. acetonitrile % provided correlation coefficients $r^2 > 0.99$ **(Table 11)**.

Knowing the linear relation between the concentrations of acetonitrile added to the mobile phase and the capacity factor, the partition coefficient of pinoresinol (K_{IAM}) can be calculated as described in Materials and Methods (**Table 12**).

The value found for pinoresinol correspond to a substance with intermediate hydrophobicity.

Table 12: Particion coefficient, K_{IAM}	
Pinoresinol	**225,581248**

11. Effect of pinoresinol on the thermotropic behaviour of model membrane composed of zwiterionic or non-lamellar phospholipids

The effect of pinoresinol in the thermotropic behavior of several phospholipids was studied, using different model membrane systems, by differential scanning calorimetry.

Firstly we observed the thermotropic behavior of model membranes composed of a zwitterionic phospholipid, which is very abundant in biological membranes – phosphatidylcholine. We chose the synthetic saturated phospholipid 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), that has two hydrocarbon chains of 14 carbons because it is an easy-to-handle phospholipid that has a very narrow and intense thermal transition, thus the monitoring of its thermotropic behaviour by DSC can be done with good sensitivity and resolution. This lipid has a pre-transition ($P_\beta \rightarrow L_\beta$) at 14 ° C and a main thermal transition from gel phase to liquid-crystalline ($L_\beta \rightarrow L_\alpha$) at around 23 ºC.

We also used the synthetic phospholipid 1,2-dielaidoyl-sn glycerol-3-phosphoethanolamine (DEPE) to study the effect of this natural compound on phospholipids which do not form lamellar phases such as the phosphatidylethanolamines (PEs). PEs are also zwitterionic phospholipids at physiological pH, relatively abundant in membranes cell and show a transition from liquid-crystalline phase to reversed hexagonal phase at higher temperatures. The main transition ($L_\alpha \rightarrow L_\beta$) of DEPE occurs at 37 ° C and the transition from liquid-crystalline to inverted hexagonal ($L_\alpha \rightarrow H_{II}$) at about 63°C.

Fig. 44 shows the DSC profiles of DMPC containing increasing concentrations of pinoresinol. The incorporation of low amounts of pinoresinol (2 mol%) induces the disappearance of the pretransition of DMPC but hardly affected the main transition. At concentrations higher than 5 mol%, DMPC transition started to broaden and shift to lower temperatures.

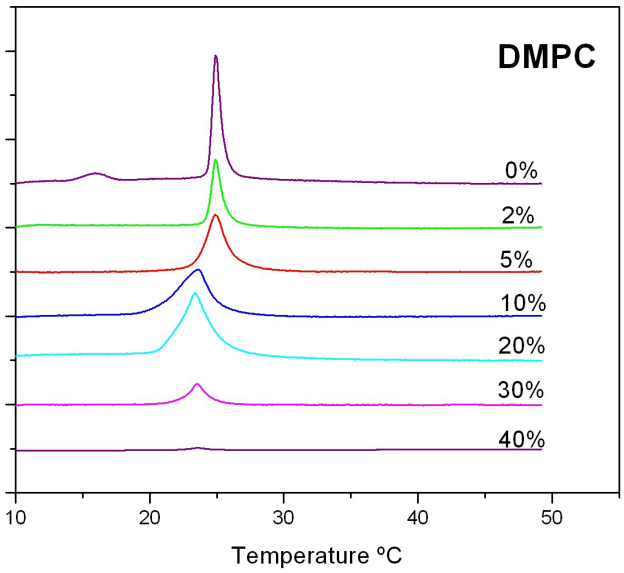
At higher amounts of Pinoresinol (10-30 mol%) the gel to liquid-crystalline broadens and gradually shifts to lower temperatures with increasing molar proportion, being barely visible at 40 mol % **(Fig. 49 A).**

The effect of pinoresinol on the thermotropic behaviour of phospholipids was also studied in model membranes composed of DEPE. As above mentioned , DEPE is a phospholipid with a main thermal transition from $L_\beta \rightarrow L_\alpha$ a 37 ºC and an additional transition from liquid-crystalline lamellar phase to non-lamellar hexagonal inverted phase ($L\alpha \rightarrow H_{II}$) at about 63 º C.

The incorporation of low amounts of pinoresinol (2 mol%) eliminated the hexagonal phase but hardly affected the main transition. Considering the transition from gel to liquid-crystalline phases, pinoresinol started to broaden the transition at concentrations of 10 mol % and above decreasing the cooperativity of the transition (**Fig. 49 B**).

At the highest concentration of pinoresinol used, i.e. 40 mol%, the main transition of DEPE was almost abolished.

A



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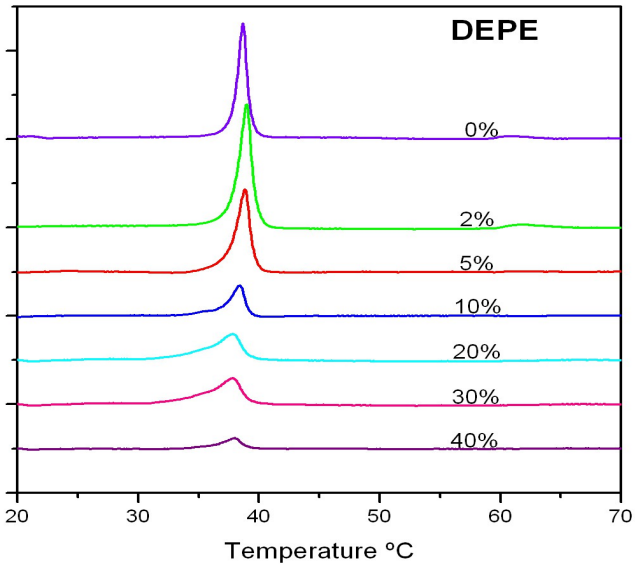


Figure 49 Differential scanning calorimetry heating thermograms for phospholipid dispersion of DMPC **(A)** and DEPE **(B)** and these phospholipids containing pinoresinol at different molar percentages.

12. Effect of pinoresinol on the thermotropic behaviour of negatively charged phospholipids model membranes

The following section describes the effect of pinoresinol on negatively charged phospholipid membranes composed of 1,2 – dimyristoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol)] (DMPG). Once more, the incorporation of low amounts of Pinoresinol (2 mol%) eliminated the pre-transition but hardly affected the main transition.

The incorporation of increasing concentrations of pinoresinol, 5% and above, induced a broadening of the main transition and a weak shift towards higher temperatures.

In contrast, the addition of 30 mol% of pinoresinol to DMPG membranes induced a significant displacement of the temperature of the main transition (Tm) to lower temperatures and at 40 mol% main transition was almost abolished (**Fig 50**).

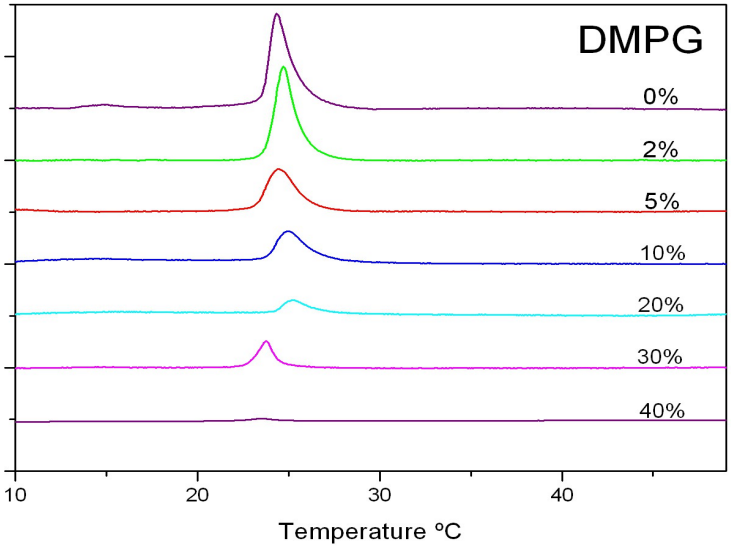


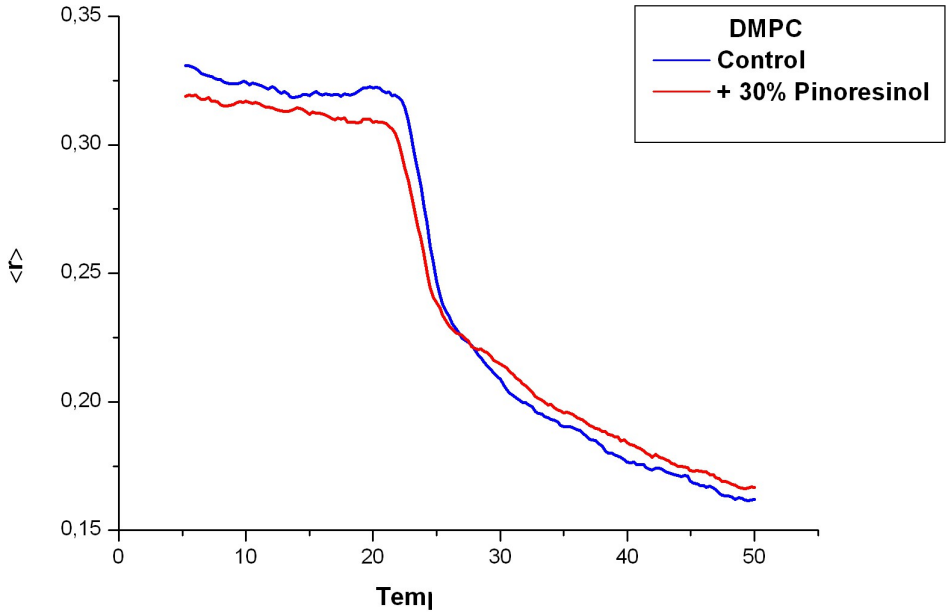
Figure 50 Differential scanning calorimetry heating thermograms for phospholipid dispersion of DMPG and this phospholipids containing Pinoresinol at different molar percentages.

13. Steady-state fluorescence anisotropy

The effect of pinoresinol on the structural and termotropic behavior of membranes composed of anionic or neutral phospholipids was also measured by steady-state fluorescence anisotropy of DPH. The fluorescence anisotropy of the probe DPH incorporated into DMPG, DMPC and DEPE was studied in the presence and in the absence of pinoresinol at 30 mol%. This probe is commonly used to estimate the fluidity and degree of order of the phospholipids locating his fluorophore into the lipidic bilayer. Figure **51 A, B and C** show the variation of the fluorescence anisotropy with the temperature of the different types of vesicles containing 30% pinoresinol.

The incorporation of pinoresinol into phospholipid vesicles didn't induce strong changes in the anisotropy values of DPH compared to the control vesicles. Nevertheless, a small decrease of the anisotropy values below the main phase transition was observed for DMPC and DMPG vesicles, whereas an increase in the anisotropy values was detected above the phospholipid transition. Although the measurement of the anisotropy values in DEPE vesicles lead to very unstable curves, a similar behavior was observed. In addition, a decrease of the phospholipid phase transition in the presence of 30% pinoresinol was observed in DMPC or DMPG vesicles, in agreement to the results obtained in the DSC experiments.

A


DMPC
Control
+ 30% Pinoresinol
<r>
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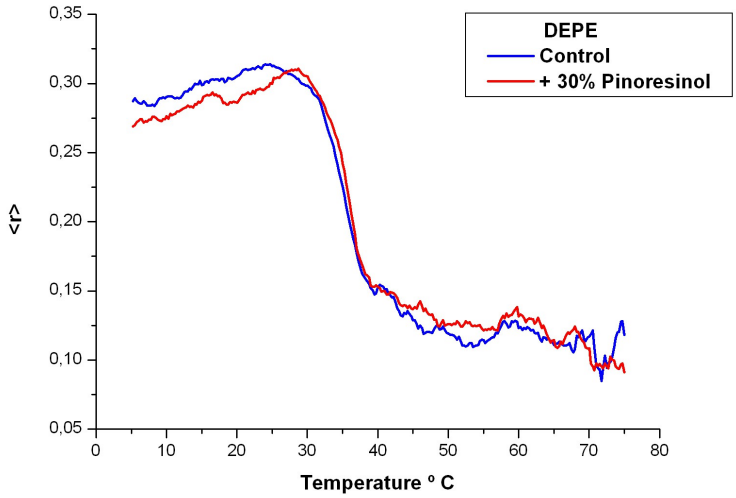
Temı

B


DEPE
Control
+ 30% Pinoresinol
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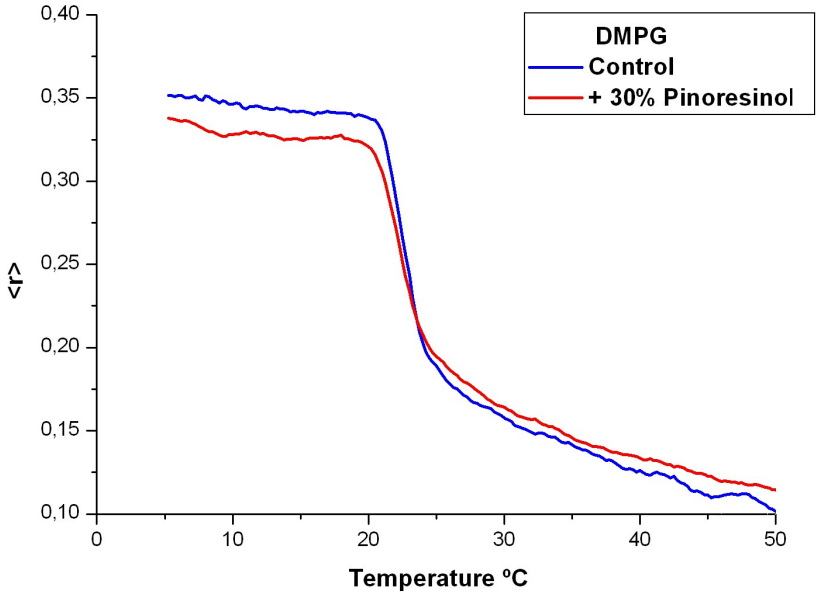
80
Temperature ° C

C


DMPG
Control
+ 30% Pinoresinol
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0,40
0,35
0,30
0,25
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0
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40
50
Temperature ºC

Figure 51 Steady-state anisotropy of DPH incorporated into (A) DMPC, (B) DEPE or (C) DMPG vesicles containing 30 mol% pinoresinol.

Discussion

Epidemiological studies have shown a lower incidence of atherosclerosis, cardiovascular diseases and certain kinds of cancer in the Mediterranean area that in other areas . Nowadays, a growing number of evidences point to the important role that extra-virgin olive oil (EVOO) plays as a crucial ingredient of the Mediterranean diet regarding their beneficial effects on health. Several studies support the hypothesis that minor components such as phenolic compounds could play a major role in the health effects of EVOO, including the prevention of chronic diseases such as cancer, obesity, diabetes, or coronary diseases .

EVOO contains different classes of phenolic compounds such as phenolic acids, phenolic alcohols, flavonoids, secoiridoids and lignans. Studies show a potential cytotoxic activity against breast cancer cells by extra virgin olive oil's phenols.

As for many plant-derived phenolics, it has been largely assumed that EVOO-derived complex phenols such as secoiridoids (that include aglycone derivatives of oleuropein, dimethyloleuropein and ligstroside, which are also present in olive fruit) and lignans (such as pinoresinol and acetoxypinoresinol) provide health benefits mainly because of their antioxidant activity .

However, the antioxidant capacity of polyphenols does not directly correlate with their efficacy in terms of anticancer activity. Moreover, plasma concentrations of EVOO polyphenols when provided in the diet are often far lower than the levels required for protection against oxidation. It could be argued that metabolites of EVOO polyphenols can reach several times higher concentrations in the bloodstream. These EVOO-derived

compounds, however, tend to have a decreased antioxidant activity compared to parent compound .

Alternatively to general mechanisms largely related to the antioxidant and/or trapping activity of oxygen radicals commonly observed in many plant-derived phenolics, recent studies have demonstrated that complex poliphenols can exert an anti-carcinogenic effect by directly modulating the activities of various types of receptor tyrosine kinases (RTKs) including several members of the HER family . Studies show that EVOO poliphenols inhibited proliferation and induced apoptotic cell death in human breast cancer cell lines bearing high levels of the tyrosine kinase receptor in HER2, an oncoprotein which is found overexpressed in ~ 15-30% of human breast carcinomas .

In particular, was demonstrated that all the fractions containing the major EVOO polyphenols, secoiridoids and the lignans can efficiently inhibit HER2 protein kinase activity by depleting the HER2 protein kinase itself. Some authors suggested that the stereochemistry of these phytochemicals might provide an excellent and safe platform for the design of new HER2-targeted anti-breast cancer drugs .

Cellular model

Cytotoxic effects of two different EVOO phenolic extract were tested on two different breast cancer cell lines: MCF7 and JIMT1, for two main reasons:

- Clinical relevance: many breast cancers are estrogen-dependent, i.e. they depend on estrogen hormones for their development and growth; MCF7 cell line, shows a positive estrogen receptor (ER) expression and suppose a model for these clinical cases. On the other hand, JIMT1 cell line, lacking expression of estrogen and progesterone receptors represents clinical cases hormone-independent.
- Different HER2-expression: HER2 represents the target of choice for the immunoliposomed systems used in the present study. It is overexpressed in 20-30% of breast and ovarian cancers and is pathologically associated with aggressive disease, increased risk of relapse and poor long-term survival . JIMT1 shows gene amplification and overexpression of HER2, but at the same time, it is resistant to the conventional therapy used for HER2-overexpressing breast cancers based on trastuzumab and another HER2 inhibiting drugs. In contrast, MCF7 cell line presents a very low HER2 expression level .

Cytotoxic assays with free drugs

The first step of the research was to evaluate the cytotoxicity of free EVOO phenolic compounds. In particular we used two EVOO varieties with different phenolic composition , a secoiridoids-rich EVOO (EVOO A) and a lignans-rich EVOO (EVOO B) in order to test both cytotoxic than cytostatic effects.

The treatments were performed for 72 hours.

The results obtained demonstrated that both cell lines were sensitive to EVOO A and EVOO B in a dose-dependent manner, but a different sensitivity was observed.

Our results further showed a close correlation between the ability of crude EVOO phenolic extracts to decrease cell viability and the expression status of HER2 in breast cancer cells (*i.e.* the effects on the cell viability were significantly more pronounced in the HER2-positive breast cancer cell lines JIMT1 than in HER2-negative cells). These findings, altogether, strongly suggest that the anti-proliferative effects shown by the phenolic compounds contained in two different varieties of EVOO may be attributed to the ability of specific phenolics to block HER2- dependent breast cancer cell proliferation processes. These findings are in agreement to previous studies that showed anti- HER2 activity of secoiridoids –rich EVOO when compared to lignans-rich EVOO . Free EVOO A resulted to be the most effective, with stronger activity in JIMT1 cells than that shown in MCF7 cells (IC50 values were approximately 20.47 µg/mL for JIMT1 and 31.84 µg/mL for JMCF7). A similar behavior of free EVOO B was observed regarding the cancer cell lines, i.e. more effective in JIMT1 cells (IC50 31.01 µg/mL) than in MCF7 cells (IC50 40.45 µg/mL).

Optimization of drugs' encapsulation into liposomes

Once the effects of EVOO compounds were analyzed in cellular models, a potential increase of their therapeutic efficacy polyphenols was tested when encapsulated into liposomes preparations. For this purpose several tests have been made to choose the best system of encapsulation. No reports on the encapsulation of EVOO polyphenols complex mixture are available in the literature.

Different parameters were changed for these optimization tests: lipid composition, incubation temperature and quantity of the compounds encapsulated. Regarding the lipid composition, two compositions were used to prepare the phospholipid vesicles: a simple

composition containing EYPC, and another composition containing EYPC/ Cholesterol (80/20), in order to mimic the percentage of cholesterol in cell membranes. The efficacy of the polyphenols encapsulation was measured with encapsulation was measured with both Folin and HPLC techniques. The results showed that most EVOO polyphenols have a higher affinity for EYPC membranes compared to EYPC/Cholesterol membranes. The presence of cholesterol decreased the affinity of several polyphenolic compounds for the membranes, probably due to the lower fluidity of the membranes. Therefore, EYPC vesicles were chosen for the subsequent experiments. Compounds such as acetoxypinoresinol and pinoresinol (lignans); luteolin and apigenin (flavones) and methyl oleuropein aglycone, oleuropein aglycone isomers I and II and 10-hidroxy oleuropein aglycone (carboxymethyl secoiridoids) showed the strongest affinity for EYPC vesicles so these compounds may be those accounting for the observed biological activity of EVOO polyphenols in the cellular systems. In contrast, polar polyphenols such as phenylethanol derivatives and decarboxymethyl secoiridoids presents less affinity for the phospholipid membranes.In addition, 37ºC were better conditions than 42ºC for encapsulation of polyphenols and 1.5% (V/V) was the optimum amount of polyphenols since higher amounts decrease the level of encapsulated polyphenols.

Cytotoxic assays with liposomed and immunoliposomated drugs

Once the liposomed preparations were optimized in order to maximize the concentration of EVOO polyphenols , we tested their effects on cellular cytotoxicity, compared to free drugs. Results showed an improvement of the efficacy of liposomed systems in all cases. We found a halving of IC50 values in the case of the treatment of JIMT1 cells with the EVOO A (10.10 µg/mL), while IC50 value decreased 26% in the case of MCF7 (23.72µg/mL). In contrast, a smaller decrease was observed when liposomed EVOO B polyphenols were used, i.e. 20% for JIMT1 cells (24.79 µg/mL) and 12.5% for MCF7 (35.41 µg/mL). These results point out that the secoiridoids-rich EVOO A polyphenol extract increases its cytotoxic capacity in a higher degree than the lignans-rich one when incorporated into liposomes. This fact also suggests that secoiridoids must be the main contributors to the cytotoxic activity of EVOO extract.

For this study, liposomes coupled to a commercial anti -HER2 antibody (trastuzumab) were used to target breast cancer cell lines which overexpress HER2 receptor. This anti-

HER2 antibody has been utilized in previous studies to successfully target HER2-overexpressing cancer cells .

Effectiveness of immunoliposomes *in vitro* was reflected in the significant reduction of IC50 values required for cytotoxic activities against the two different cancer cell lines. JIMT1 and MCF7 were sensitive to free drugs, especially to EVOO A with IC50 values ranging from 20 to 31 μg/mL mM for free EVOO A and 31 to 40 μg/mL for EVOO B .

Cellular responses to the treatments based on anti-HER2 immunoliposomes showed a reduction in the IC 50 value further than that obtained for regular liposomes.

Immunoliposomated EVOO A showed a reduction in JIMT1 cells of almost 70% in comparison to IC50 values of JIMT1 cells treated with free EVOO A, while a reduction of 45% was observed in the case of immunoliposomated EVOO B.

In MCF7 cells the reduction in IC50 value was weaker with a decrease of 49% using immunoliposomed EVOO A and 27% with immunoliposomed EVOO B in comparison to the IC50 values of cells treated with their respective free polyphenols extracts.

These results suggest that immunoliposomed systems showed higher cytotoxic activity in the two cell lines utilized probably due to their higher selectivity. However, some considerations must be stated. Our results showed that secoiridoid-enriched EVOO polyphenol extract (A) was the most cytotoxic in both cell lines. Nevertheless, its efficacy was extremely enhanced in JIMT1 cells when this extract was incorporated into immunoliposomes, what was probably due to the higher HER2 expression of these cells compared to MCF7 cells. In addition, the fact that EVOO A was less effective in MCF7 cells compared to JIMT1 cells in all cases points out that secoiridoids may target different proliferation pathways in JIMT1 cells. Immunoliposomed EVOO B was also more cytotoxic in JIMT1 than in MCF7 cells but it showed lower capacity than EVOO A, probably due to its lower content in secoiridoids.

Interaction of pinoresinol with model membranes

Studies on the molecular interaction of compounds and model membranes require significant amounts of purified compounds that usually are difficult to purify using chromatographic techniques. Therefore, a commercially available source must be used. Among the bioactive secoriridoids and lignans found in EVOO extracts only oleuropein and pinoresinol were commercially available. Oleuropein has been thoroughly study both

in cancer cells and model membrane studies , then we selected pinoresinol to perform studies on its incorporation in model membranes.

Lignans are of considerable pharmacological interest because they are known to have many biological properties, such as antioxidant, antitumor, antiviral, antibacterial, insecticidal, fungistatic and anti-platelet activities, in addition to protective effects against coronary heart disease .

Studies demonstrated that pinoresinol-rich EVOO extracts have potent chemopreventive properties and specifically upregulate the ATM–p53 cascade .

In the present study, lignans were used vehiculized in phospholipid vesicles to treat cancer cells, hence, it must be expected that this compound may interact with cancer cell membranes and affect their biophysical properties. Since this compound shows intermediate polarity, it's presumed that can get through cell membranes in some extent reaching intracellular targets.

The interactions of drugs with biological membranes affect the delivery of drugs to the target sites within the body. Usually, a drug has to cross several membranes in order to enter the target location. As a result, the optimization of the delivery of drugs requires understanding of the interactions of drugs with biological membranes. Knowledge of these interactions is also of prime importance when predicting vehiculization, adsorption, distribution, metabolism, and excretion (ADME) properties of drugs already in the early phases of drug discovery process.

The most common physicochemical property used in the prediction of drug-membrane interactions is the lipophilicity of a drug, which is usually expressed as logP, the logarithm of the partition coefficient between two immiscible solvents. Traditionally, the partition coefficient has been determined using n-octanol and water. However, the ability of the octanol−water partition coefficient to describe drug partitioning in membranes has been questioned due to the major differences in the biophysical properties of octanol and phospholipid cell membrane. Due to this, alternative approaches, have been developed.

In addition, the association of pinoresinol with biological membranes may promote changes in the membrane physical properties and therefore modulate membrane-dependent biological processes. Some authors have previously studied the interaction of some olive oil phenols such as oleuropein , hydroxytyrosol , hydroxytyrosol acetate,3,4-dihydroxyphenylelenolic acid and 3,4-dihydroxyphenylelenolic acid dialdehyde but no previous studies about the lignan pinoresinol are reported .

We have studied the association, localization and effect of pinoresinol in biological membranes composed of zwiterionic or anionic phospholipids.

The pinoresinol phospholipid/water partition coefficient has been calculated by IAM-PLC methodology; these immobilized artificial membrane columns mimic the lipid environment of a fluid cell membrane on a solid matrix. The method is very fast, it allows a relatively high throughput, requires only small amounts of solutes, and impurities as well as degradation products seldom interfere with the procedure .

Ottiger et al. reported a better results about determination of partition coefficient when log k'_{IAM} vs mole fraction was used . The partition coefficient K_{IAM} obtained by HPLC-IAM showed a certain affinity for phospholipid membranes and an intermediate hydrophobicity of pinoresinol. The log K'_{IAMw} value obtained for pinoresinol was 1.33, that is similar to log K'_{IAMw} values of (*RS*) propanolol and diazepam calculed by the same method . The effect of the incorporation of pinoresinol on the physical properties of phospholipid model membranes was studied by using different biophysical techniques.

DSC results obtained in this work using neutral (DMPC, DMPE) or negative phospholipids (DMPG) showed significant changes of the $L_\beta \rightarrow L_\alpha$ phase transition.

A significant transition broadening was noticed from DSC thermograms of DMPC vesicles containing 5% pinoresinol and above. At higher concentrations, of pinoresinol, the onset temperature of the gel-to-liquid crystalline phase transition decrease gradually indicating a good mixing between the phospholipid and pinoresinol, which reveals a molecular interaction between this compound and the phospholipids, despite its high polarity. At higher concentrations, i.e 30-40%, pinoresinol was able to almost abolish the main transition of DMPC. In contrast, immiscibility was observed between pinoresinol and the phospholipid at the fluid line. A similar behaviour was observed for the unsaturated and non-lamellar phospholipid DEPE, although the interaction between this phospholipid and pinoresinol seemed to be weaker than that observed for DMPC. In the case of the anionic phospholipid DMPG, Tm of the phospholipid did not decrease significantly upon the increase of pinoresinol, and then no strong interaction was present. Nevertheless, a narrowing of the transition peak was observed at 30% pinoresinol probably indicating the presence of a complex between the phospholipid and pinoresinol with a 2:1 ratio. At higher concentrations of pinoresinol, the transition of DMPG was completely abolished.

The effect of pinoresinol on the lipid order of the same phospholipid vesicles was studied by fluorescence anisotropy. The incorporation of 30% pinoresinol decreased anisotropy

DPH values in the gel phase of the two phospholipids (DMPC and DMPG) indicating a decrease of the lipid order and packing. In contrast, DPH anisotropy values slightly increased in the fluid phase of the phospholipids revealing a mild increase in the lipid order. A similar effect was observed in DEPE vesicles but anisotropy signal was anisotropy measurements were quite unstable probably due to the unsaturated character of the phospholipid that induced instability of the DPH probe.

Conclusions

1. EVOO polyphenols are efficiently encapsulated into phospholipid vesicles. In particular, acetoxypinoresinol and pinoresinol (lignans); luteolin and apigenin (flavones) and methyl oleuropein aglycone, oleuropein aglycone isomers I and II and 10-hidroxy oleuropein aglycone (carboxymethyl secoiridoids) show strong affinity for EYPC vesicles. Encapsulation was less efficient in EYPC vesicles containing cholesterol.

2. Secoiridoid-rich EVOO A induced a more significant cytotoxic effect than lignan-rich EVOO B in the two breast cancer cell lines. JIMT1 breast cancer cells were more sensitive than MCF7 cells to both EVOO A and EVOO B, which might be related to the higher HER2 receptor expression of JIMT1 cells and the capacity of virgin oil polyphenols to interfere HER2- dependent breast cancer cell proliferation processes.

3. Optimum condition of incorporation of EVOO extracts in liposomes is using EYPC and incubing for 1 h at 37ºC with a lower content in extract, (i.e. 3% EYPC more 1.5% (V/V) of extract.

The incorporation of EVOO A and EVOO B into EYPC liposomes significantly improved their cytotoxic effect on human breast cancer cell models. This decrease was enhanced for EVOO A compared to EVOO B, indicating that bioactive polyphenols exerting the cytotoxic activity are probably more related to the secoiridoid group and these compounds reach their target easily when incorporated into liposomes.

4. The incorporation of EVOO A and EVOO B into immunoliposomes improved its cytotoxicity on JIMT1 breast cancer cells through the decrease of their IC50 values at an average of 36-30 %decrease compared to liposomes, and an average of 70-45 %decrease compared to free compounds. The improvement was less pronounced in MCF7 breast cancer cells, indicating that its proliferation capability is more resistant to the anticancer activity of secoiridoids and probably independent of HER2 pathways.

5. The bioactive lignan pinoresinol shows intermediate polarity but interacts significantly with phospholipid model membranes. This compound modify the physical properties of neutral (PC) and anionic (PG) membranes at molar concentrations starting at 5-10 mol%, most probably by interacting with phospholipid head groups. In addition, pinoresinol increases the fluidity of membranes in the gel phase and increases lipid order in fluid membranes. These effects may account to the biological activity observed for pinoresinol and other similar lignans.

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List of Abbreviations

ADCC antibody-dependent cellular cytotoxicity

AUC area under curve

BC breast cancer

BRCA1 and **BRCA2** Breast Cancer genes 1 and 2

Chol cholesterol

CL cardiolipin

COX cyclooxygenase

D200M 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide (PEG 2000]

DDS Drug delivery system

DMEM Dulbecco's modified Eagle medium

DMPC Dimyristoylphosphatidylcholine

DMSO Dimethylsulfoxide

DNA deoxyribonucleic acids

DPH 1,6-Diphenyl-1,3,5-Hexatriene

DPPC dipalmitoylphosphatidylcholine

ECD extracellular domain

EDTA Ethylenediaminetetraacetic acid

ER estrogen receptor

ERT postmenopausal estrogen therapy

ETOH Ethanol

EYPC egg yolk phosphatidylcholine

EVOO Extra Virgin Olive Oil

FBS fetal bovine serum

FDA Food and Drug Administration

HER2 human epidermal growth factor receptor 2

HPLC High performance liquid chromatography

HRT Hormone replacement therapy

IGF-1R insulin-like growth factor-1 receptor

IC50 inhibitory concentration 50%

IL Interleukin

ILs immunoliposomes

LCIS lobular carcinoma in situ

MAPK mitogen-activated protein kinases

MLVs multilamellar vesicles

LUVs unilamellar vesicles

MPS mononuclear phagocyte system

MTT (3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)

NF-k_ nuclear factor-kappa _

NK natural killer

PBS phosphate buffered saline

PC phosphatidylcholine

PDI polydispersity index

PE phosphatidylethanolamine

PEG Polyethylene glycol

PI3K Phosphatidylinositol 3' kinase

PR progesterone receptor

ROS reactive oxygen species

RT room temperature

RTKs receptor tyrosine kinases

SM sphingomyelin

SUVs small unilamellar vesicles

TBS Tris-buffered saline

TGFα Tumor Growth Factor alpha

THIS Histidine buffer

UICC Union Internationale Centre Cancer

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